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TIME-RESOLVED SUPPRESSION OF FLUORESCENCE IN RAMAN SPECTROMETRY BY CONTINUOUS WAVE LASER AMPLITUDE MODULATION AND PHASE-SENSITIVE DETECTION.

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ABSTRACT

Raman spectroscopy applied to biological systems is often dominated by interfering fluorescence background. In this report Raman scattering of benzene in the presence of strong fluorescence was obtained via phase sensitive detection upon sinusoidally modulating the continous wave laser beam at tens of MHz. Extended detectability of the 992 cm^{-1} frequency was obtained, but the signal enhancement is greatly dependent on the experimental measuring time.

1. INTRODUCTION

The luminescence background often forms a major obstacle in obtaining good quality Raman and resonance Raman spectra of aro-

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matic molecules in condensed media. Especially in biological systems the Raman scattering can be obscured by trace amounts of fluorescent biomaterial or impurities present. Generally there are several methods to circumvent the interference of fluorescence. The methodology can be divided into spatial and temporal resolution of Raman signals. Spatial resolution from isotropic fluorescence is obtained by coherent Raman techniques, in which the Raman signal has been generated as a coherent beam. Examples are Coherent Anti-Stokes Raman Scattering (CARS)[1], Stimulated Raman Spectroscopy[2] and Inverse Raman Spectroscopy[3].

The alternative approach of background reduction by time resolution is based on the difference between the duration of fluorescence (ns-time region) and that of the Raman effect (ps-time region). In this particular technique Raman photons can be discriminated in time against fluorescence photons by the use of either mode-locked[4] or synchronously pumped dye lasers[5] in combination with high repetitive gated photon counting. This technique has been critically appraised recently[6].

Other experimental approaches are based on frequency and amplitude modulation of the laser beam. In case of frequency modulation[7,8] the narrow Raman frequencies are modulated synchronously, whereas the broad fluorescence background remains unaffected. With phase sensitive detection the Raman signals can be extracted from the constant background. Amplitude variation of continuous wave (CW) lasers is, in fact, a variant of the time discriminating method, since the fluorescence is shifted in phase and is reduced in amplitude relative to the excitation. Phase fluorimeters for measuring fluorescence decay rates are based upon this phenomenon[9,10].

In this report we make use of an amplitude modulated CW laser beam in conjunction with a conventional Raman spectrometer system and a commercial high frequency lock-in amplifier. It has

been shown that picosecond time resolution can be obtained in this way by a careful choice of modulation frequency[11]. A description of the set-up is given, which has the particular advantage that conventional and fluorescence suppressed Raman spectra can be simultaneously collected allowing for a critical comparison. The 992 cm^{-1} frequency of benzene in the absence and presence of a fluorescent flavin solute was used to monitor the effects. In principle the same instrument can be used as a phase fluorimeter with photon counting sensitivity.

2. EXPERIMENTAL SECTION

2.1 REAGENTS

3-methyllumiflavin, synthesized as described in reference 12 and dissolved in benzene (Merck, analytical grade), was used to generate the fluorescent background. The compound is abbreviated as MLF.

2.2 EXCITATION METHOD

A Coherent Radiation CR 18UV argon ion laser was used as a continuous light source at 488 nm, output power 800 mW. The laser beam was modulated using an electro-optic modulator model 22S from Coherent Associates (Figure 1). Using this modulator as described earlier[13], the light tranmission as a function of the applied voltage is a sinusoidal curve with maxima of transmission of 80 % and minima of transmission of about 0.2 %. With a bias supply the voltage was set for minimum transmission. After applying a sinusoidal modulation signal the laser light was modulated at twice the electrical modulation frequency. The sinu-

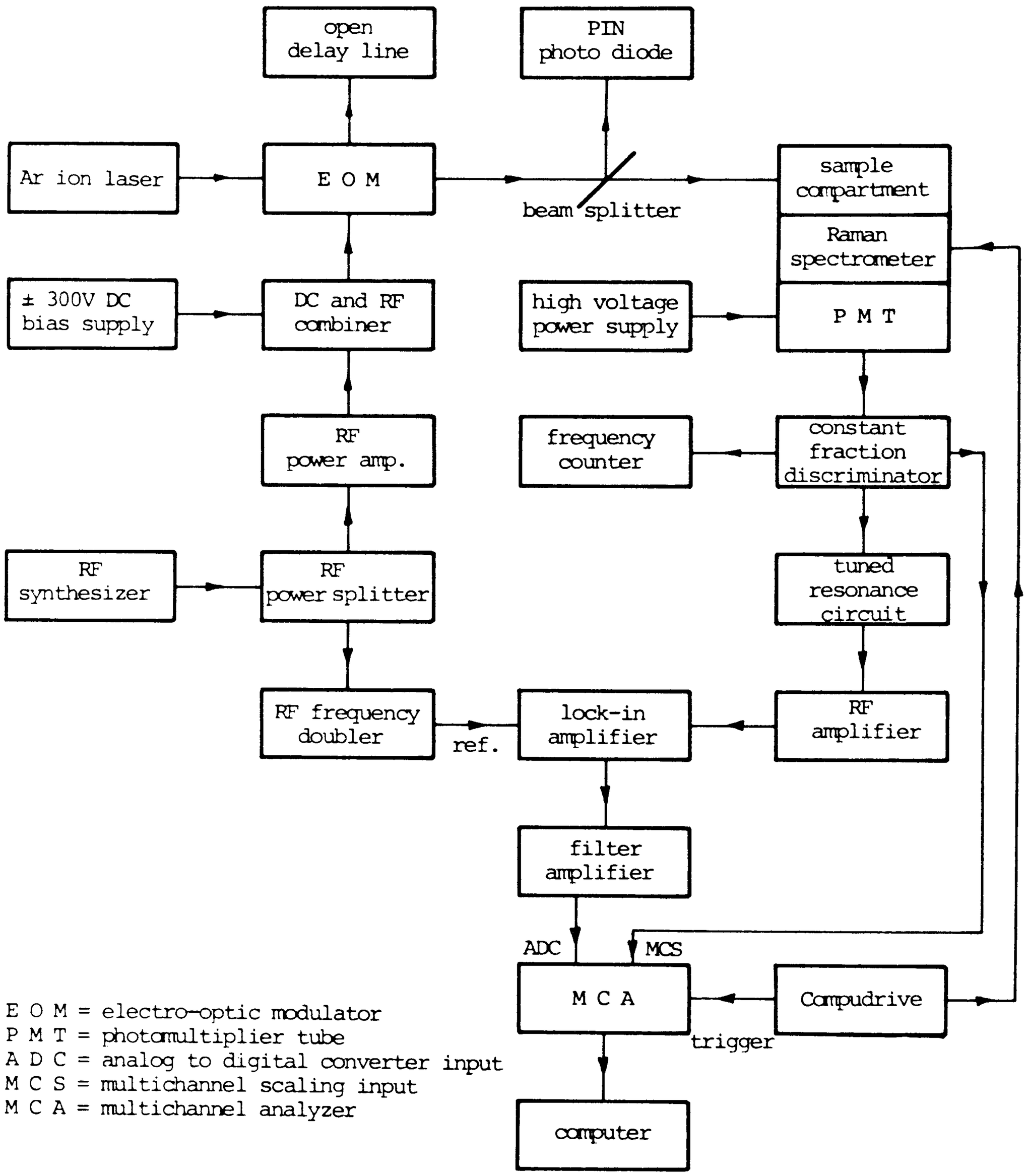

FIGURE 1

Block diagram of the experimental set-up.

soidal radio frequency (RF) modulation signal (23 MHz) came from a synthesizer (Schomandl ND 60M), amplified via a power splitter (ENI model PMU 12-2) with a 10 W broadband amplifier (ENI model 310 L).

The output of this amplifier was combined with the direct current (DC) bias voltage using some passive components in a shielded box. Because of the maximum output power of the RF power amplifier of 10 W in 50Ω a maximum modulation voltage of about 70 V peak-to-peak could be applied. In the curve of light transmission versus bias voltage applied to the modulator the difference in voltage of two points of maximum transmission was about 175 V at 488 nm.

For adapting the impedance mismatch between the RF drive amplifier (50 Ω) and the modulator (16.6 Ω delay line concept), the modulator was terminated using a 50Ω open delay line. The length of this delay line (Ortec model 425) was tuned, thus adapting the effective impedance at the given frequency. The quality of the modulation was monitored using a battery powered PIN photodiode (HP type 5082-4203) and an oscilloscope (Tektronix model 485). The light power (20 mW) was controlled using a variable neutral density filter.

2.3 DETECTION METHOD

The samples were in a 0.2 ml quartz cuvette. The Spex model 1403 Raman spectrometer was controlled via a Spex Compudrive, also delivering the different trigger signals for the detection electronics. The detection bandwidth of the spectrometer was set to 5 cm^{-1}. The detection photomultiplier (PMT) was a Philips PM 2254B cooled at -20°C in a Products for Research model TE 104RF housing. The single photon pulses from the anode of the

PMT were discriminated using a Canberra model 1428A constant fraction discriminator for optimum time resolution.

The time resolution was mainly determined by both transit time fluctuations in the PMT and light path differences in the monochromator. The two positive five volt pulse outputs were used for data storing via the multichannel scaling (MCS) input of the multichannel analyzer (MCA, Nuclear Data model ND66), and for monitoring the photon frequency (Hewlett Packard model 5328A counter) to determine the desired attenuation of the excitation light.

The well defined negative 0.8 V and 10 ns pulses were fed into a resonance circuit. For that purpose a 50 Ω and 46 MHz bandpass filter was used out of the ^{2}H lock channel of a Bruker 300 MHz ^{1}H nuclear magnetic resonance spectrometer. In this way every photon pulse induced a decaying sinusoidal signal, its phase being determined by the time of arrival of the photon at the PMT cathode. Photon frequencies of up to several hundred thousands Hz were used. The output signal of the resonance circuit was amplified (Ortec model 574) and fed into the input of a PAR model 5202 lock-in amplifier (0.1 - 30 MHz).

The use of a discriminator output instead of the photon current pulses from the anode to excite the resonance circuit is advantageous. It gives the possibility to detect simultaneously with single photon counting and synchronous detection and last but not least, a lot of spurious signals and also gain fluctuations of the PMT were avoided.

The reference signal 46 MHz for the lock-in amplifier came from the synthesizer via a power splitter and a frequency doubler (Mini Circuits model GK3). Frequency doubling in the light and RF pathways was useful to get a low background interference in the room at the detection frequency of the lock-in amplifier. The output of the lock-in amplifier was filtered and amplified using

a home-built low frequency amplifier and then fed into the analog to digital converter (ADC) input of the MCA. The MCA was able to handle four input jobs simultaneously. During the experiments described two channels were used. One channel was a zero dead time MCS input gathering the photon counts from the discriminator. The other channel was the ADC input, sampling the amplified output signal of the lock-in amplifier in a list mode. The channel stepping was controlled by the Compudrive.

3. RESULTS AND DISCUSSION

The results of the experiments are shown in Figures 2 and 3. Figure 2 shows the two simultaneous recordings of the 992 cm^{-1} frequency of the benzene solvent. The upper trace is the synchronously detected one. This signal has been scaled to its peak value (8 V) and has been given an offset of 1.8 V to ensure that during the scan the output voltage of the lock-in amplifier falls within the dynamic range of the ADC of the MCA.

The lower trace is the accumulation of photon counts into the channels of the MCA during the scan. In this particular case the peak value has been scaled to 200,000 counts. Both detection methods result in equally strong signals, although the background noise level of the synchronously detected scan is somewhat larger. This is mainly due to the chosen time constant (0.3 s) of the filter amplifier as compared to the 1 s photon counting integration time per channel.

In Figure 3 simultaneously collected recordings of benzene doped with 20 μM MLF are shown. The upper trace is the output signal of the lock-in amplifier and was detected in phase with the (previously recorded) benzene signal. Again, this signal has been scaled to its maximum value (8 V) and has been offset by 4 V.

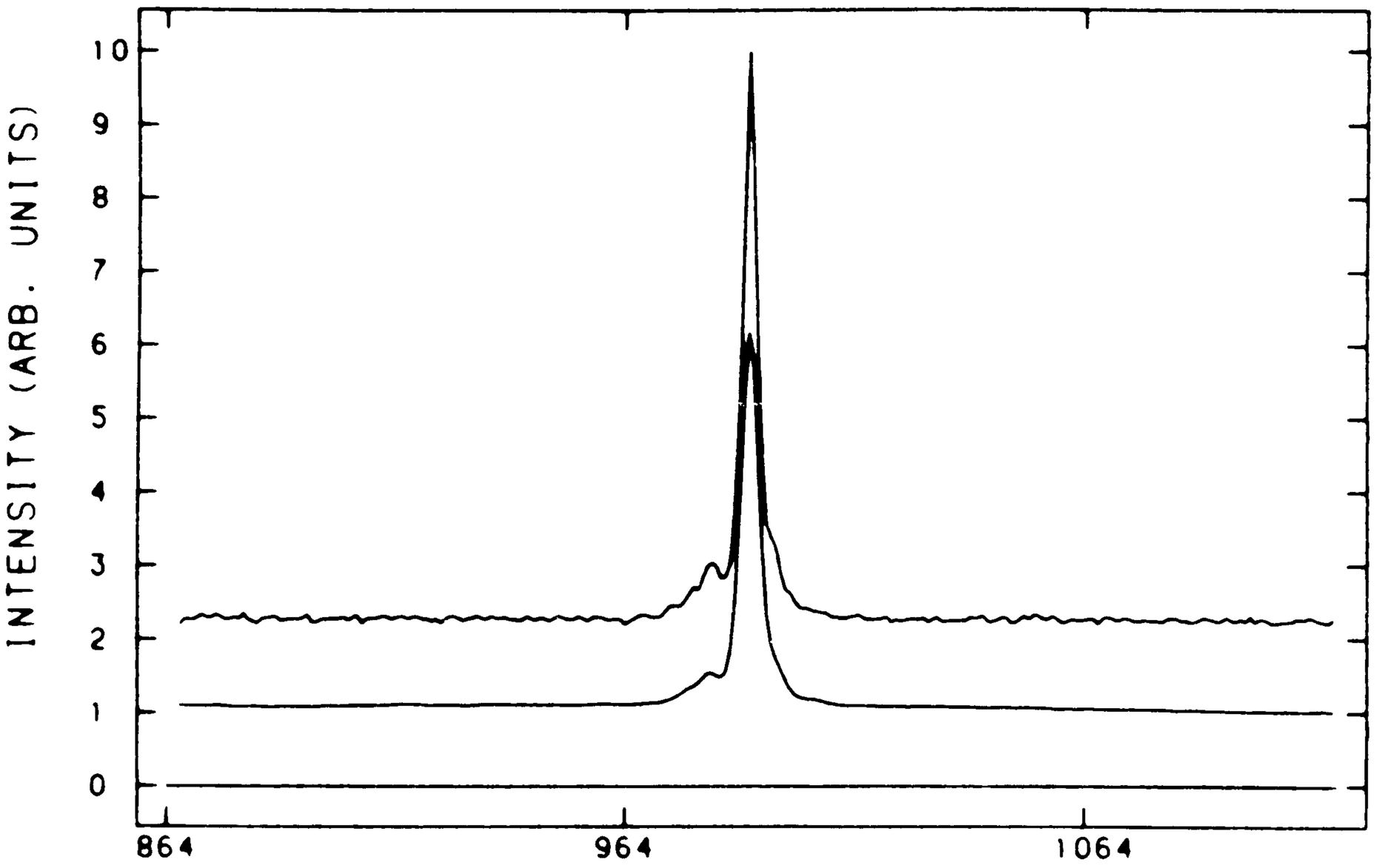

FIGURE 2

Raman spectrum of benzene either synchronously detected (SD, upper trace) or with photon counting (PC) detection (lower trace)

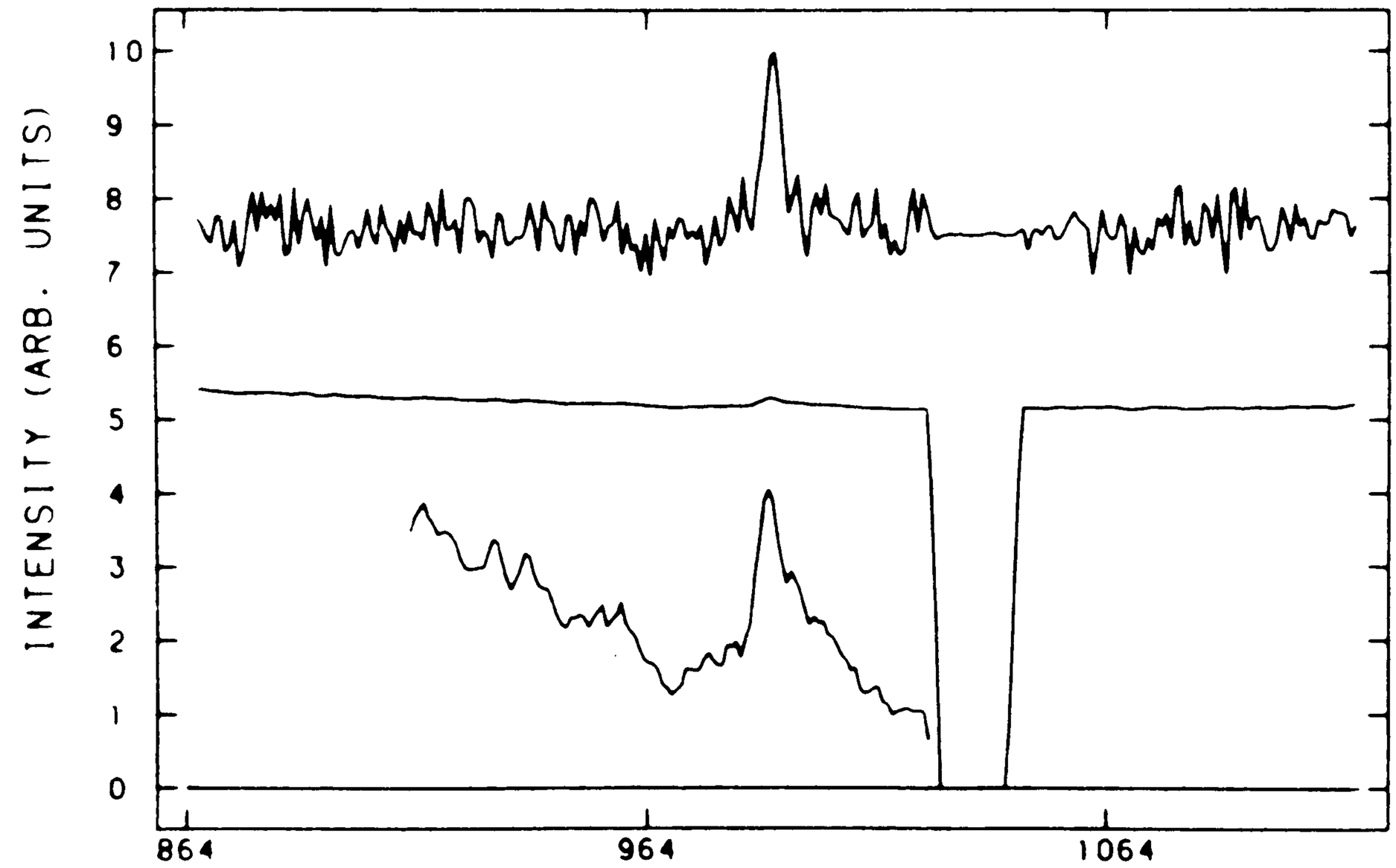

FIGURE 3

Raman spectrum of benzene doped with 20 μM MLF, SD scan upper trace and PC scan lower trace. The inset is a scaled up fragment of the PC scan (see text). In the case of a shut-off of the laser light the PC spectrum goes to zero and the SD spectrum remains unaffected but the noise level is decreased.

The second trace originates from photon counting. Counts were normalized to one million to facilitate easy comparison. The benzene signal has been swamped out by the huge fluorescent background in contrast to the synchronously detected result. The inset in Figure 3 shows an enlargement of the benzene 992 cm^{-1} intensity by subtraction of the fluorescence background followed by normalization to 50,000 counts. The decrease of the signal as compared to the spectrum of pure benzene can be well explained by a relative decrease in excitation power owing to light absorption by the flavin.

Shutting off the laser light (Figure 3) is illustrative since the photon counting signal reduces to zero as expected, but the signal of the synchronous detector remained at the same offset level, however with much less noise. The fluorescence lifetime of the flavin derivative is in the order of 5 ns (for accurate determinations see references 13 and 14).

The noise on the curves recorded via pure photon counting originates from photon counting statistics and also from minor laser intensity fluctuations. In the case of the synchronously detected emission the noise is not only dependent on the photon statistics, but also on the effect of the fluctuating time of arrival in the nanosecond region, resulting in noise signal from out of phase detected photon events. With a low-pass filter this noise can be greatly reduced.

In fact this is a principal difference as compared to other techniques of fluorescence suppression based on temporal resolution using a gating time window. When the fluorescence decay time constant is shorter than about one nanosecond, almost complete overlap in time of fluorescence and Raman radiation is caused by transit time spread in the detection chain. In synchronous detection mode with application of a 90° phase shift between Raman scattering and fluorescence the Raman signal can be completely

recovered at the expense of measuring time. With respect to that it should be noted that the relative phase difference between emission and excitation light is also an average of different photon events. We want to illustrate this point by considering the effect of two events with opposite phases.

Let us denote Raman photon events at the input of the lock-in amplifier by:

$$R = r.\cos \omega t.e^{\frac{-t}{\tau}}$$

(r = amplitude, ω = angular frequency and τ = damping time constant of the 46 MHz resonance circuit). The transit time spread of the detection system causes phase shifts ϕ, but the sum S_r of two different, opposing events will be:

$$S_r = r.e^{\frac{-t}{\tau}} [\cos(\omega t-\phi) + \cos(\omega t-\phi)]$$

$$S_r = 2r.e^{\frac{-t}{\tau}} \cos\omega t.\cos\phi$$

with ϕ the parameter representing the detection transit time spread. For the out of phase detected fluorescence events $F = f.\sin\omega t$ the same relationship holds true:

$$S_f = f.e^{\frac{-t}{\tau}} [\sin(\omega t+\phi) + \sin(\omega t-\phi)]$$

$$S_f = 2f.e^{\frac{-t}{\tau}} \sin\omega t.\cos\phi$$

Thus independent of the value of ϕ in the in and out of phase detected signals S_r and S_f can be separated when sufficient time is spent to average the output signal of the synchronous detector. It is not easy to predict the desired experimental time to extract Raman signals from fluorescence background with a certain accuracy because it depends on the yield of Raman scattering and fluorescence but also on the transit time spread in the detection system and on the modulation frequency required for a

90° phase shift. We have measured a standard deviation of the transit time spread in the total detection system of about 800 ps and this system response was very reproducible.

It was rather easy to get a two times better signal to noise (S/N) ratio of the 992 cm^{-1} Raman peak of benzene doped with 20 μM MLF, given the scan time of only 256 seconds for the 256 cm^{-1} wavenumber range of Figures 2 and 3.

The S/N ratios of the mentioned Raman peak were defined as the ratio of 2.5 times peak height and the peak to peak value of the noise, taken over a wavelength range of about 50 cm^{-1}. In Figure 2 the signal to noise ratio of the upper trace was 130, of the lower trace 266. In Figure 3 the S/N ratios were 7 and 4 respectively. The latter figure was corrected for the descending baseline.

Measurement of Raman spectra from biological samples, which are often characterized by ps-fluorescence lifetime components, requires higher light modulation and lock-in detection frequencies. Because of the long time desired to average out the effects of transit time spread of the detection system a very long measuring time should then be anticipated.

4. ACKNOWLEDGEMENT

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