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A Compact Violet Diode Laser–Based Fluorescence Lifetime Microscope

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We have constructed a time-correlated single-photon counting (TCSPC) microscope system using a pulsed violet-laser diode for measuring fluorescence lifetimes below 1 ns. These compact; cool, and fast-pulsed laser diodes are much more suitable for lifetime measurements than the traditional mode-locked lasers used in the past. The laser source (LDH-400, PicoQuant GmbH) is mounted on an optical table above the microscope, and the laser beam is directed into an Olympus BX-60 microscope by means of a dichroic beamsplitter set at 45 degrees. The system operates in backscattering mode with the fluorescence emission passing back through the dichroic beamsplitter and focused into a 100 mm focal length monochromator with a PMT detector. This prototype instrument is compact (~80 × 70 × 70 cm) and is nearly fully computer controlled by means of a SPC-730 (Becker & Hickl) PC card. We include preliminary results showing the instrument response function (IRF) of the system, and some of the factors have been adjusted to minimize the temporal width of the IRF. The instrument has been validated using a series of standard fluorophores at different emission wavelengths.

KEY WORDS: Fluorescence; lifetime; laser diode; microscopy.

INTRODUCTION

Fluorescence microscopy is an important measuring tool in the life sciences because of its inherent sensitivity and the development of very specific fluorescent probes. The majority of fluoresce microscopy systems in use tend to be based on steady-state techniques, partly due to cost and technology issues. Steady-state systems, however, suffer from problems such as photobleaching, probe concentration variation, and scattering artifacts that affect fluorescence intensity measurements [1]. time-resolved fluorescence microscopy (TRFM) systems, on the other hand, are much less sensitive to these effects [2]. Systems for TRFM, which usually utilize either frequency or time domain measurement methods, have been reported, although most of these instruments incorporate sophisticated laser systems as the excitation source, which require high maintenance and make also them expensive to acquire [3–5]. Semiconductor laser diodes offer the advantage of small size and low costs, and recently a TRFM system based on a 635-nm diode has been reported [6].

We present a TRFM system based on a pulsed violet laser diode using the TCSPC method. This is a simple and versatile system primarily intended for analysis of microscopic petroleum fluid inclusions and biological systems.

INSTRUMENTATION

The microscope system is depicted in Fig. 1 and consists of a 402-nm violet laser diode, which can be

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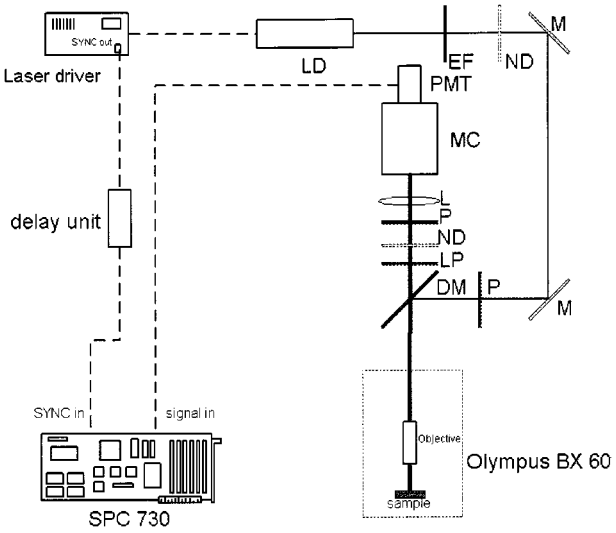

Fig. 1. Setup for time-resolved fluorescence microscopy system. **EF:** Excitation filter; **ND:** neutral density filter: **M:** mirror; **P:** polarizer; **DM:** dichroic beamsplitter; **LP:** longpass filter; **L:** lens; **MC:** monochromator; **LD:** violet laser diode.

pulsed up to 40 MHz (LDH-400 & PDL-800B, PicoQuant, Germany). The laser has a minimum pulse width of ~55 ps and a maximum power output of 1.5 mW at 40 MHz. An output trigger signal from the laser driver is connected to a nanosecond delay unit (Model 7800-7, FastComTec, Germany) and from there is connected to a TCSPC board (SPC-730 Becker and Hickl, Germany). The pulsed laser emission is filtered (D 405/40 AHF analysentechnik, Germany) and then steered using a pair of mirrors through a film polarizer (25% transmission, Edmund Scientific, stock no. P38-493) to preserve beam polarization. The beam is then directed downward using a dichroic beamsplitter (425 DCXR, AHF) into an Olympus BX-60 microscope via a standard three-way binocular head (U-TR30, Olympus). The laser beam is then focused onto the sample, and the fluorescence emission is collected by the microscope objectives (standard Olympus units). The fluorescence emission passes through the dichroic beamsplitter and through a slider arrangement, which holds a cutoff filter (GG 435LP, AHF) to minimize scattered laser light entering the detection system. The slider also holds neutral density filters for taking IRF measurements. A film polarizer was also fitted to help minimize a direct reflection from the rear face of the dichroic beamsplitter. The fluorescence is then focused using a 50-mm focal length lens onto the entrance slit of a 100-mm focal length motorized monochromator (Model 9030, Sciencetech Inc., Canada) fitted with a 1200-line grating blazed at 650 n*m*. The emission is detected using a metal package photomultiplier tube (model 7353, Hamamatsu), the output of which is fed to the TCSPC board. Lifetimes are fitted by reconvolution using the Fluofit software package (PicoQuant).

RESULTS AND DISCUSSION

In Fig. 2, curve A depicts a typical IRF curve measured using this system and shows a number of peaks. The most intense peak in the IRF trace is due to the scattering from the Teflon sample, but there is also pair of peaks preceding this peak. The first peak is approximately 2 ns earlier than the main peak and ~1.5 % of its intensity. This time interval corresponds to a pathlength of approximately 60 cm, which is equal to the pathlength between the dichroic beamsplitter and the sample. This indicates that this peak is due to a reflection from the dichroic beamsplitter itself, possibly from the rear face. The second peak, which precedes the main peak by 1 ns and is ~30% of the intensity of the main peak, is due to a reflection from the field lens within the binocular head of the microscope through which the laser beam passes. This lens is a standard lens for ocular viewing and as such has no antireflection coating to minimize laser reflections at this wavelength. These reflections can be minimized by incorporating a film polarizer above the dichroic beamsplitter and rotating it such that it is orientated 90 degrees relative to the polarization of the laser emission. In Fig. 2, curve B, a significant drop in the intensity of both peaks relative to the scattering peak can be observed, proving that both peaks are due to laser reflections (which are highly polarized) and not due to a

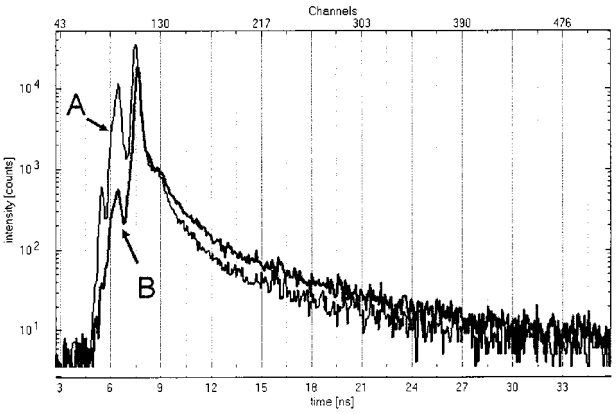

Fig. 2. IRF measured without a polarizer (A), and with a polarizer oriented at 90 degrees with respect to polarization of the excitation light, placed above the dichroic mirror (B). IRF was measured using white Teflon as a scattering target. Monochromator was set at 405 nm; the longpass filter and both entrance and exit slits were removed. The laser was run at 10 MHz rep. rate with 50% power.

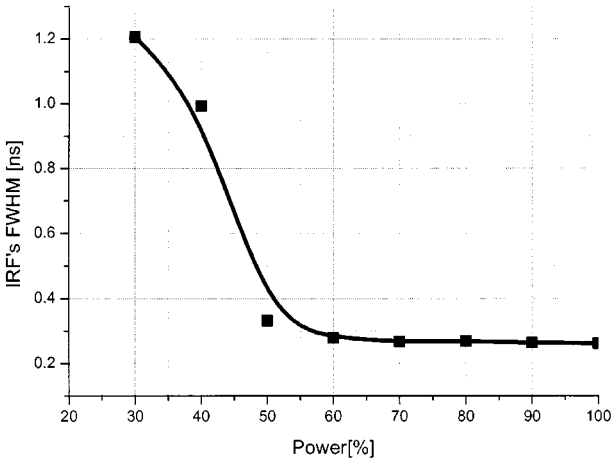

Fig. 3. FWHM of the IRF vs. the power output of the LDH-400 diode laser. Measuring conditions were 10-MHz repetition rate, Teflon target.

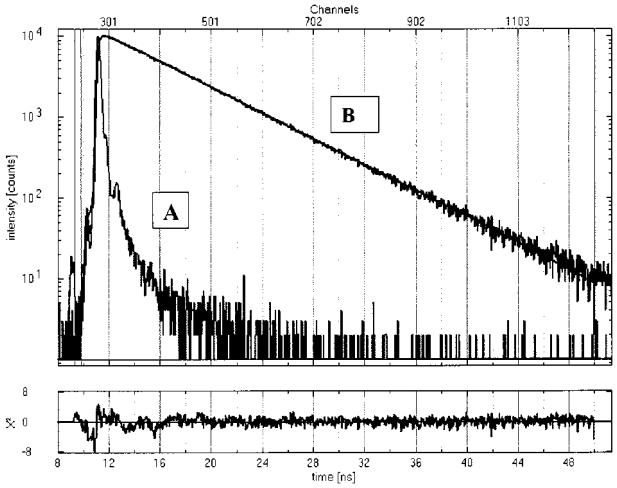

Fig. 4. The IRF curve (A), fluorescence decay curve (B) for 10^{-5} M HPTS in phosphate buffer (0.1 M) detected at 550 nm. The residuals plot is for a one exponential deconvolution fit of the decay curve.

scattering effect from the sample (which would be more randomly polarized). The first peak is reduced to ~0.4%, and the second peak to ~6%, of the intensity of the main peak, after correction for the 25% transmission of the film polarizer.

Figure 3 shows the dependence of the full-width half maximum (FWHM) of the IRF with increasing laser power. Once the power setting on the laser diode is set above 50%, the IRF has reached a minimum FWHM of ~270 ps. When the laser power is set below ~30%, no lasing occurs and there is just a broad, comparatively long-lived spontaneous emission. From 30 to 50%, the lasing increases, but there is a considerable amount of the spontaneous emission, which leads to a large FWHM that gradually decreases. The minimum value of the IRF FWHM is limited by the temporal resolution of the metal package PMT used in this system. These detectors are, however, compact and moderately priced so are ideal for most lifetime applications.

8-Hydroxypyrene-1,3,6-trisulphonic acid trisodium salt (HPTS) was chosen as a test fluorophore because previous studies in our laboratory had shown that a relatively strong absorbance around 400 nm and that it had a single exponential lifetime decay. Figure 4 shows the decay curve recorded at 500 n*m* and the residuals for a one exponential fit. The largest errors occur at the beginning of the fit, where the spurious reflections occur in the IRF trace. The recovered lifetime for HPTS is 5.3 ns, as shown in Table I, where the decay plot was fitted to a series of models. These values agree with previous studies in this laboratory using a cuvette-based system [7].

CONCLUSIONS

The relatively simple TRFM system demonstrated here requires no modification of the microscope, because all the excitation and emission components are mounted on an optical table above the trinocular unit. This type of approach, which can be applied to most infinity corrected microscopes allows for the measurement of sub-nanosecond lifetimes over the 450–850 n*m* emission range. Using

Table I. Results for one, two, and three exponential fits to the decay plots shown in Fig. 4. The pre-exponential terms (α_i) are given in percentages; the terms in brackets are the errors as calculated by support plane analysis.

α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)	χ^2
100	5.334	–	–	–	–	1.71
–	(± 0.028)	–	–	–	–	
31	5.342	−69	0.03	–	–	1.43
(± 0.32)	(± 0.14)	(± 11)	(± 0.164)	–	–	
32	5.362	1	0.723	−67	0.044	1.40
(± 0.015)	(± 0.019)	(± 0.41)	(± 0.464)	(± 2.2)	(± 0.002)	

this system we have measured the lifetime of HPTS to be 5.3 ns, which is in good agreement with experimental [7] and literature values [8]. The fit quality is, unfortunately, poor, which is due to two spurious reflections originating from a field lens in the trinocular unit and from the dichroic beamsplitter. These reflections only occur in the IRF measurements, because the longpass filter removes any scattered laser light from the fluorescence signals. The spurious reflections can be reduced in intensity by the use of crossed polarizers, but this unfortunately limits this system in its current configuration to the study of isotropically emitting samples. We are currently investigating the use of picosecond dyes for IRF measurements, which would eliminate this reflection problem, allowing the analysis of anisotropic samples.

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