

SHORT COMMUNICATION

# Frequency-domain fluorescence microscopy with the LED as a light source

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## Summary

We describe a frequency-domain lifetime fluorometer based on a microscope and a modulated light-emitting diode (LED) excitation source (370/460 nm), which operates in the frequency range 120 Hz–250 MHz. We collected multi-frequency phase and modulation fluorescence responses from cellular areas as small as 10–15  $\mu\text{m}$  in diameter. We also collected fluorescence lifetime data from cells stained by a lipophilic coumarin sensitized europium fluorophore, Coum-Eu, with a millisecond lifetime, and Ru(bpy)<sub>2</sub>phe-C<sub>12</sub>, with microsecond lifetime. Nanosecond lifetimes from native nuclei stained with SYTO 14 and SYTO 16 probes were measured as well. We demonstrate that a simple LED excitation source can, for many applications, successfully replace complex and expensive laser systems, which have been used for cellular frequency-domain lifetime measurements. As the LEDs are very stable with low noise, it will be possible to image even smaller sample areas using brighter LEDs. With availability of modulated LEDs emitting at several wavelengths covering almost the entire visible spectrum it is easy to assemble a system for the fluorophore of choice. The ability to select an excitation source for a given fluorophore and low price make such an excitation source even more practical.

## Introduction

Time-resolved fluorescence methods, especially implementations for lifetime fluorescence microscopy, have required complicated and very expensive instruments (Despa *et al.*, 2000). There has been a lack of a simple, affordable and

stable light source that would be bright enough for microscopy measurements and that would also cover a lifetime range from nanoseconds (ns) to milliseconds (ms). In the frequency domain, lifetime measurements typically require expensive pulsed laser systems or the use of light modulators. The pulsed lasers are most optimally used at MHz repetition rates and are most suitable for ns or faster time range. Even though it is possible to use them at slower repetition rates, the resulting loss of light output makes them impractical. In the case of modulators, it is possible to cover a ms to ns time window, but the electro-optical light modulators reject more than 90% of the input light, are difficult to adjust, and require highly collimated light beams such as lasers.

Recently there has been a growing interest in the use of light-emitting diodes (LEDs) as a light source for both steady-state and time-resolved fluorescence spectroscopy (Hauser & Tan, 1993; Hauser *et al.*, 1995; Sipior *et al.*, 1997; Harms *et al.*, 1999; Olson *et al.*, 1999; Szmactinski & Chang, 2000). LEDs as an excitation source cover the whole visible range and offer excellent stability, light-noise levels, power efficiency and economy. Recently LEDs have become available with an emission in the blue and UV spectral regions (370 nm), which can be modulated from DC up to 300 MHz (Szmactinski & Chang, 2000). This frequency is high enough for measurements of ns lifetimes.

In this paper we describe a frequency-domain microscope-based fluorometer with a UV/VIS LED excitation capable of measuring fluorescence decays from the ms to the ns timescale. This is, to the best of our knowledge, the first report describing usage of LEDs for microscopical frequency-domain measurements. We have used this instrument to examine localized fluorescence decays from 3T3 fibroblasts stained by a ms sensitized Eu chelate, a  $\mu\text{s}$  ruthenium-based metal–ligand complex (Ru-MLC) and ns SYTO dyes.

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## Materials and methods

### Chemicals

MOPS, 7-amino-4-(trifluoromethyl) coumarin (Coum), diethylene-triamine-pentaacetic-acid-dianhydride (DTPA), triethylamine, europium chloride ( $\text{EuCl}_3$ ),  $\text{D}_2\text{O}$ , dodecylamine, 1,3-dicyclohexyl-carbodiimide (DCC), *N*-hydroxysuccinimide (NHS), ruthenium chloride, 2,2'-bipyridine (bpy), amino-phenanthroline (phe- $\text{NH}_2$ ), trifluoroacetic acid (TFA), thiophosgene, acetonitrile and DMF were purchased from Aldrich (Milwaukee, WI). SYTO 14 and SYTO 16 dyes were purchased from Molecular Probes (Eugene, OR).

### Synthesis

The  $\text{Ru}(\text{bpy})_2\text{phe-NH}_2$  was a gift from Dr J. Dattelbaum. The metal–ligand complex (MLC) was reacted with 500  $\mu\text{L}$  thiophosgene in 500  $\mu\text{L}$  acetone for 3 h. Both the solvent and the thiophosgene were dried under  $\text{N}_2$  and resulting isothiocyanate was reacted with excess ( $10\times$ ) of dodecylamine in DMF for 12 h. The product was purified by reverse-phase high performance liquid chromatography (HPLC). The sensitized chelator for Eu was made following the procedure of Li & Selvin (1997). Typically, 5–10 mg of the sensitizer (Coum) was dissolved in less than 500  $\mu\text{L}$  of anhydrous DMF containing an equimolar amount of dry triethylamine. The solution was added dropwise with shaking to a small volume (less than 250 mL) of dry DMF containing  $1.5\times$  moles of DTPA. The reaction was allowed to proceed overnight. Products were purified on a reverse-phase HPLC C18 column using 0.1% TFA and 100% acetonitrile containing 0.05% TFA. To link an allyl chain to the chelator, the sensitized DTPA was first activated by conversion to the NHS ester using standard protocols. Typically, coum-DTPA (2–3 mg) was treated for 6 h with  $1.1\times$  moles of DCC and NHS each, in a small volume of dry DMF. The activated material was reacted overnight with  $2\times$  moles of dodecylamine. The product was purified on a C18 reverse phase HPLC column using 0.1% TFA and acetonitrile containing 0.05% TFA.

### Samples

Mouse fibroblasts (3T3 Swiss albino, ATCC number CCL-92) were grown at 37 °C and a 5%  $\text{CO}_2$  atmosphere in glass-bottomed dishes (MatTek Corp., Ashland, MA) containing Dulbecco's modified Eagle's medium with 10% calf serum. The cells were ringed in buffer and stained with 1  $\mu\text{M}$  solutions of SYTO 14 and SYTO 16 dyes. In some cases the cells in the dishes were fixed in 70% ethanol (4 °C, at least 30 min). After rinsing, the 3T3 cells attached to the cover slip were treated with 100  $\mu\text{L}$  of a 0.1 mM solution of either Ru MLC or Coum-Eu fatty acid in 20% methanol for 20 min. Then the cells were washed with clean buffer and imaged.

### Fluorescence measurements

All measurements were performed on a standard Zeiss Axiovert 135TV inverted epi-fluorescence microscope with a broad-band noncoherent LED excitation. The ms Coum-Eu fluorophore was excited by a Nichia (Tokushima, Japan) NSHU550E UV LED with a peak emission centred near 370 nm. The  $\text{Ru}(\text{bpy})_2\text{phe-C}_{12}$  and SYTO dyes were excited by a Nichia NSPB500S high power blue LED with peak emission around 460 nm. The typical long-wavelength fluorescent tails of the UV and blue LEDs were suppressed by a Schott glass 7–59 filter and a 505-nm barrier interference filter inserted in the excitation path, respectively. The epi-illumination was accomplished using a Zeiss C-Apochromat  $40\times/1.2$  W (water immersion) objective lens. For measurements of Coum-Eu we used a combination of Zeiss FT 395 dichroic beam splitter and a narrow band Chroma (Brattleboro, VT) ( $615 \pm 7$ ) nm emission interference filter, model D615/14 M. For SYTO dyes we used a Chroma Q505LP dichroic beam splitter and an Omega Optical 560RDF55 ( $560 \pm 55$ ) nm IF filter.  $\text{Ru}(\text{bpy})_2\text{phe-C}_{12}$  was measured with a Chroma Q505LP beam splitter and an Omega 635DF55 IF filter ( $635 \pm 23$ ) nm.

For practical purposes the multifrequency fluorescence decays were measured in the so-called 'fast-scan' mode. The phase and modulation data were first collected at all frequencies for a sample and then the procedure was repeated with a slide containing a suitable reference compound. This approach allowed fast data acquisition and eliminated sample handling and position uncertainty associated with repetitive changing of sample and reference compounds. A small drop of Rhodamine B on a cover slip was used as a reference.

The fluorescence images were taken with an open excitation field diaphragm by redirecting the fluorescence signal to the second camera port equipped with an image intensifier model C5825 (Hamamatsu, Bridgewater, NJ) and a PXL digital camera (Photometrics, Tucson, AZ).

Intensity decays were analysed in terms of the multi-exponential model,

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i), \quad (1)$$

where  $\alpha_i$  are the pre-exponential factors,  $\tau_i$  the decay times, and  $\sum \alpha_i = 1.0$ . The fractional intensity of each decay time is given by

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \quad (2)$$

The mean lifetime ( $\bar{\tau}$ ) of a multiexponential decay is given by

$$\bar{\tau} = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \quad (3)$$

The values of  $\alpha_i$  and  $\tau_i$  were recovered by non-linear least

squares by minimizing  $\chi_R^2$  (Gratton *et al.*, 1984; Lakowicz *et al.*, 1984)

$$\chi_R^2 = \frac{1}{\nu} \sum \left( \frac{\phi_\omega - \phi_{c\omega}}{\delta\phi} \right)^2 + \frac{1}{\nu} \sum \left( \frac{m_\omega - m_{c\omega}}{\delta m} \right)^2 \quad (4)$$

In this expression,  $\phi_\omega$  and  $m_\omega$  are the measured values of the phase ( $\phi$ ) and modulation ( $m$ ) at the light modulation frequency  $\omega$ . The values of  $\phi_{c\omega}$  and  $m_{c\omega}$  are the calculated values for assumed values of parameters  $\alpha_i$  and  $\tau_i$ . The values of  $\delta\omega = 0.4$  and  $\delta m = 0.007$  are the assumed experimental uncertainties in the phase and modulation, respectively, and  $\nu$  is the number of degrees of freedom of the analysed data set.

## Results

### Apparatus

A block scheme of the microfluorometer with LED excitation is shown in Fig. 1. The instrument was based on a Zeiss Axiovert 135TV epifluorescence microscope. Köhler illumination was accomplished by coupling the excitation light from a frequency-modulated LED to the microscope via a high numerical aperture quartz collimator located on the input port of the microscope. The size of the illuminated area was chosen by a light-field diaphragm located in the excitation path. For conventional imaging and sample alignment the diaphragm was opened in order to obtain

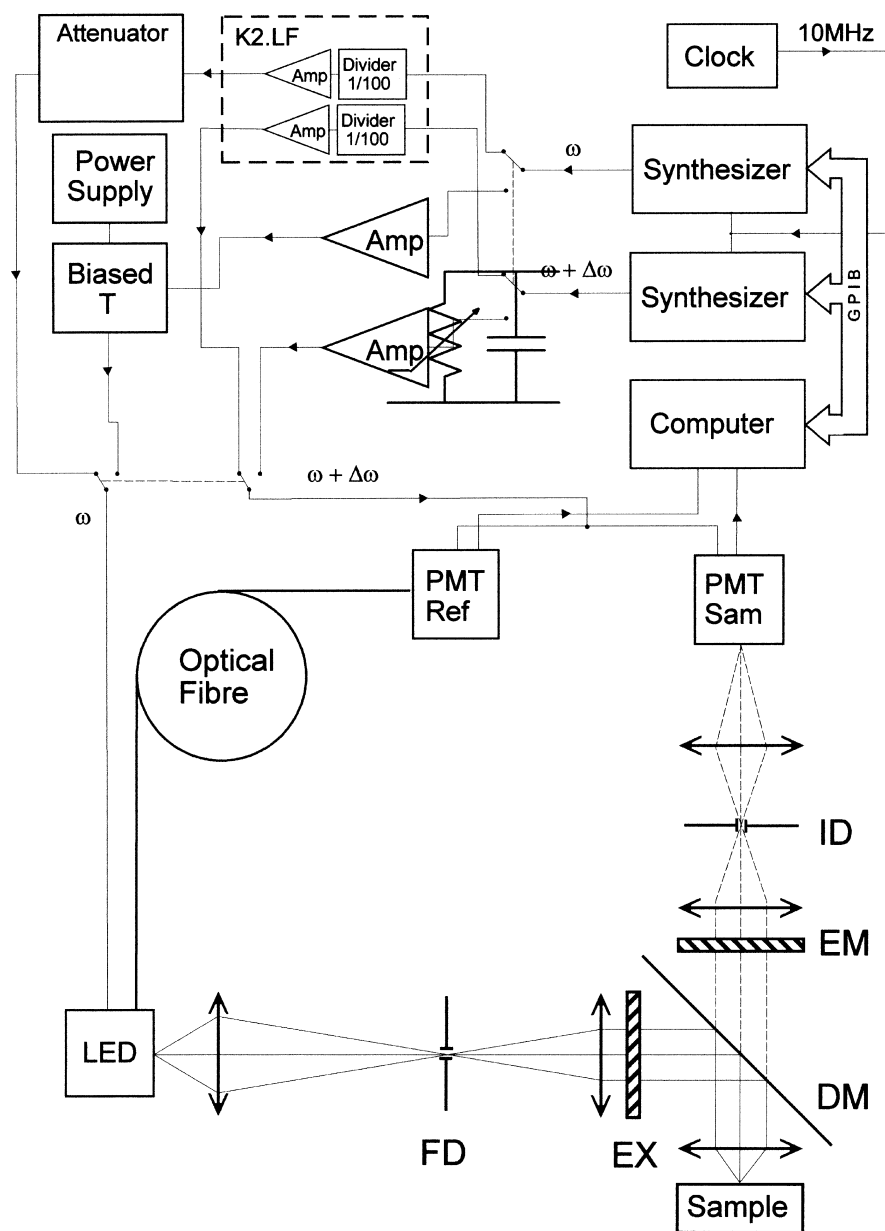


Fig. 1. Block scheme of the frequency-domain time-resolved microfluorometer with LED excitation. FD = excitation field diaphragm, ID = image plane diaphragm, EX = excitation filter, EM = emission filter, DM = dichroic mirror.

the maximum field of view. The illumination area was minimized for the localized frequency-domain measurements. The second optically conjugated diaphragm was located in the image plane of one of the available camera ports. Its size matched the image size of the illuminated area in the sample, which was set to be about 15  $\mu\text{m}$  in diameter. With the positions of both the diaphragms fixed, the area of interest within the sample was chosen by scanning the  $x$ - $y$  microscope stage.

The detection part of the instrument was based on an ISS-K2 (ISS Inc., Champaign, IL) frequency-domain instrument. A modulated detector PMT was mounted on the output port of the microscope. In order to minimize diffraction losses, the light emerging from the output pinhole was collected by a high numerical aperture lens and focused on the photocathode of the PMT. A small fraction of the excitation light was coupled to the quartz light-guide and directed to the reference PMT of the ISS K2 instrument.

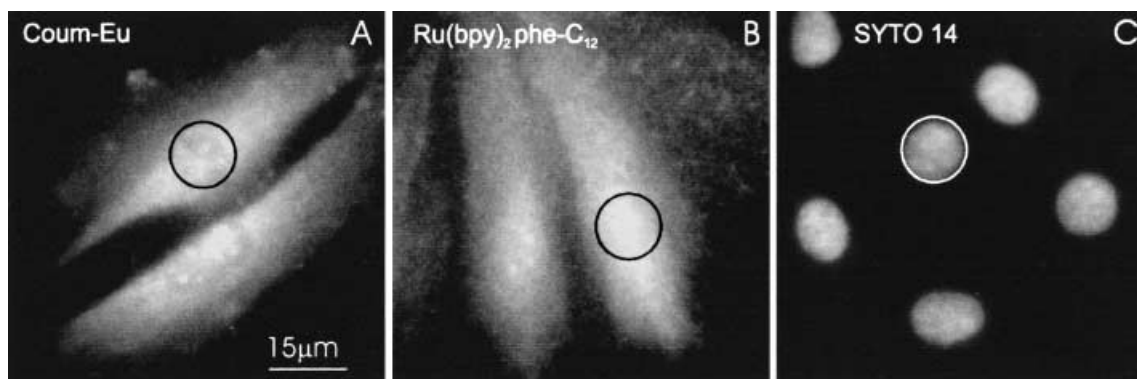
For ms measurements the UV LED was driven directly by the attenuated modulator driver output of the K2.LF low frequency module (ISS Inc) driven by the output of the frequency synthesizer (Marconi Instruments, Allendale, NJ, model 2022D). Because the module contains a 1/100 frequency divider, we could with this set-up obtain a useful modulation depth in the continuous frequency range 120 Hz–2 MHz. Lower modulation frequencies would require a different frequency synthesizer and one would need to operate the system at a lower cross-correlation frequency. The high frequency cut-off was caused by the bandwidth of the low frequency module rather than by the diode, which can be modulated to at least 250 MHz (unpublished results; Szmanski & Chang, 2000). In the  $\mu\text{s}$  and ns experiments the LED was biased at 70 mA with an LDX-3412 Precision Current Source (ILX Lightwave, Bozeman, MT) through a bias tee (Picosecond Pulse Laboratories, Boulder, CO, model 5580). The modulation current was supplied directly by an output of the power

amplifier (ENI, Rochester, NY, model 403LA) driven by a synthesizer at  $-8$  dBm (Marconi, model 2022D). Under these conditions we were able to modulate the LED with reasonable modulation depth in the frequency range 40 kHz–250 MHz. The modulation bandwidth was limited on the low-frequency side by a low-frequency cut-off of the power amplifier. At high frequencies the modulation was limited by a combination of a diode modulation bandwidth and a decreased frequency response of the PMT.

### Cell measurements

Figure 2 shows intensity images of 3T3 fibroblasts stained with the ms sensitized lanthanide probe Coum-Eu, the  $\mu\text{s}$  Ru(bpy)<sub>2</sub>phe-C<sub>12</sub>, and the ns nucleic acid stain SYTO 14. Visual inspection of the images shows good contrast between the non-specific background and the stained cells. The cells stained with the lipophilic probes Coum-Eu and Ru(bpy)<sub>2</sub>phe-C<sub>12</sub> exhibited fairly uniform fluorescence from the cell body, which was expected for probes designed to stain membrane structures. Native cells stained with SYTO 14 exhibited fluorescence only in the nuclear region without non-specific staining of other cellular components. The data collection area, marked by a circle, was approximately the size of a nucleus and allowed low resolution fluorescence measurements within one cell or discrimination between signals from individual cells from a larger ensemble.

Figure 3 shows the microscope-based frequency-domain fluorescence decays of Coum-Eu, Ru(bpy)<sub>2</sub>phe-C<sub>12</sub>-stained cells and of SYTO 14 and SYTO 16 from the cell nuclear locations indicated in Fig. 2. The phase and modulation data can be fitted by a multiexponential decay model and the results are presented in Table 1. The random nature of error distribution indicates the absence of systematic artefacts. For comparison we also measured frequency-domain fluorescence decays of Coum-Eu and Ru(bpy)<sub>2</sub>phe-C<sub>12</sub>



**Fig. 2.** Intensity images of 3T3 fibroblasts stained by lipophilic Coum-Eu (A), Ru(bpy)<sub>2</sub>phe-C<sub>12</sub> (B) and nuclear stain SYTO 14 (C). Circles indicate areas from where the fluorescence signal was collected. Cells shown in (A) and (B) were ethanol-fixed; (C) shows living 3T3 fibroblasts.

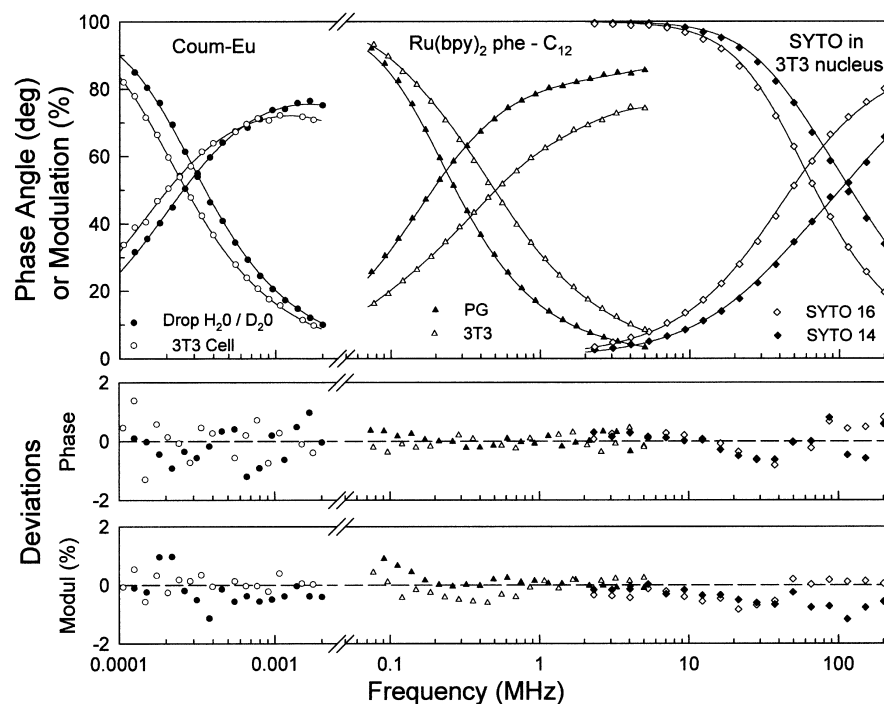


Fig. 3. Microfluorometric frequency-domain decays of Coum-Eu in a drop of 50% D<sub>2</sub>O (○) and in an ethanol-fixed 3T3 fibroblast (50%D<sub>2</sub>O) (●); Ru(bpy)<sub>2</sub>phe-C<sub>12</sub> in a drop of propylene glycol (▲) and in an ethanol-fixed 3T3 fibroblast (△); and SYTO 16 (◇) and SYTO 14 (◆) in native nuclei of 3T3 fibroblasts. Areas from which fluorescence was collected is marked in Fig. 2. Fluorescence of Coum-Eu was excited by a UV LED near 370 nm and emission was selected by a (615 ± 7) nm bandpass IF filter. Emission of Ru(bpy)<sub>2</sub>phe-C<sub>12</sub> and SYTO dyes was excited by a blue LED near 450 nm, and fluorescence was selected by a broad band IF filter centred at 635 nm and 560 nm, respectively.

in a small drop. In a pure solvent the probes exhibited almost monoexponential fluorescence decays with small contribution (< 2%) of a short component (Table 1). Coum-Eu and Ru(bpy)<sub>2</sub>phe-C<sub>12</sub> decay with ms and  $\mu$ s lifetimes, respectively, consistent with published values for similar classes of fluorophores (Evangelista *et al.*, 1988; Takeuchi *et al.*, 1995; Terpetschnig *et al.*, 1995a, 1995b; Szmackinski *et al.*, 1996). Fluorescence decays from the cells show

Table 1. Lifetimes of fluorophores in solution and cellular environments

Compound	Lifetime	$\alpha_i^a$	$f_i^b$	$\chi_R^{2c}$
Coum-Eu (drop, 50%D <sub>2</sub> O)	0.01 ms	0.72	0.02	1.5
	0.78 ms	0.28	0.98	
Coum-Eu (fixed 3T3 cell, 50%D <sub>2</sub> O)	0.24 ms	0.02	0.03	1.1
	0.50 ms	0.80	0.02	
Ru(bpy) <sub>2</sub> phe-C <sub>12</sub> (PG)	1.05 ms	0.18	0.95	1.1
	0.06 $\mu$ s	0.11	0.007	
Ru(bpy) <sub>2</sub> phe-C <sub>12</sub> (Fixed 3T3 cell)	1.02 $\mu$ s	0.89	0.993	1.6
	0.04 $\mu$ s	0.34	0.04	
SYTO 16 (native 3T3 nucleus)	0.32 $\mu$ s	0.51	0.49	1.6
	1.04 $\mu$ s	0.15	0.47	
SYTO 14 (native 3T3 nucleus)	4.01 ns	1.0	1.0	1.6
SYTO 14 (native 3T3 nucleus)	1.26 ns	0.67	0.42	1.7
	3.51 ns	0.33	0.58	

<sup>a</sup> Fractional amplitudes, <sup>b</sup> fractional intensities, <sup>c</sup> reduced sum of squared residuals for  $\delta m = 0.007$  and  $\delta \phi = 0.4$  deg.

higher heterogeneity, as expected from a heterogeneous environment. Nanosecond lifetimes of SYTO 14 and 16 in the nucleus are also reported in Table 1.

## Discussion

In the preceding sections we described a microscope-based frequency-domain fluorometer with a convenient LED excitation and demonstrated that such an instrument is usable for measurement of lifetimes from ms to ns time ranges.

For microscopy measurements signal levels are always an important issue. The advantages of the modulated LED excitation compared to pulsed-lasers is obvious for ms and  $\mu$ s fluorescence lifetime experiments. In order to measure lifetimes accurately, fluorescence has to decay significantly in the time-window between excitation pulses. Measurements of long lifetimes therefore require low pulse repetition frequencies. For cavity-pumped dye lasers or Ti:Sapphire lasers with a pulse selector, the average output power scales proportionally or nearly proportionally with the repetition frequency and often falls below the usable limit at the low frequencies required for measurements of  $\mu$ s or ms lifetimes. This is especially true for blue or UV excitation when second harmonics of the laser output have to be used. However, in the frequency-domain experiments the periodically modulated LEDs give almost the same mean optical output power over decades of modulation frequencies. This modulated light output can be comparable with the output of the unmodulated LED, because modulated diodes can operate at

elevated peak currents. In practical terms the LEDs we used produce milliwatts of optical output, whereas a typical Ti:Sapphire laser with a pulse selector running at 100 kHz produces less than 0.1 mW in blue. It is therefore obvious that the multifrequency frequency-domain approach can utilize all advantages of periodically modulated LEDs. We have noticed that the low optical noise of LEDs allows for reliable measurements of signals several times weaker than those needed with a pulsed dye-laser excitation or with electro-optically modulated CW laser beams. In practice, even for low fluorescence signals, the signal-to-noise ratio of the LED excited fluorescence was often superior.

In our current set-up, the spatial resolution, i.e. the minimal size of the excitation and data collection area, is limited by the available fluorescence signal. We have shown that in wide-field illumination mode the signal levels allow for moderate spatial resolution of the order of 10–15  $\mu\text{m}$  even with relatively weak UV LEDs. Improvement of the spatial resolution can be achieved with brighter diodes or in narrow-field illumination mode. The LEDs used in the present study have an output of about 1 mW in UV and 6 mW in blue. As LEDs with a higher optical output are becoming commercially available, we can expect significant improvements in both the resolution and the capability to analyse smaller spots.

It is not our intention to suggest that an LED light source can replace lasers for all applications in frequency-domain microscopy. For example, multiphoton microscopy will always need ps and fs lasers such as Ti:Sapphire. Similarly, when it is necessary to collect an excitation spectrum, only a tunable laser will be useful. However, for a great many frequency-domain microscopical measurements, we believe that high quality fluorometric lifetime data with moderate spatial resolution can be obtained with LED excitation in a lifetime range from ms to ns. The technique is applicable even for relatively dim fluorophores, such as Ru-MLCs. The advantages of the modulated LED excitation become pronounced at low modulation frequencies and make LEDs a viable alternative light source for ns and especially  $\mu\text{s}$  to ms lifetime measurements.

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