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Hyper Rayleigh Scattering Yields Improved Response Function in Analysing 2-Photon Excited Fluorescence

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An accurate instrumental response function is needed to conclusively deconvolute fluorescence data based on time-correlated single-photon counting (TCSPC) and multiphoton excitation. Routinely the response function is measured as Rayleigh scattering (RS) from a colloidal solution, even if the excitation is a multiphoton event. Present work demonstrates that a response function obtained as hyper Rayleigh scattering (HRS) provides a better choice for deconvolution of 2-photon excited fluorescence decays. The 1- and 2-photon response functions were monitored as RS and HRS from colloidal gold particles at 800 and 400 nm, respectively.

KEY WORDS: Two-photon-excited fluorescence; hyper Rayleigh scattering; response function; time-correlated single-photon counting.

Today a wide range of methods and applications in chemistry, biology, and medicine are based on time-resolved fluorescence spectroscopy [1]. Recent development of ultrafast laser sources enables using routinely excitation pulses shorter than 100 fs. One important use of femtosecond lasers in fluorescence spectroscopy is to create multiphoton excitation (MPE). Until recent studies of MPE using the time-correlated single-photon counting (TCSPC) technique [2,3], most studies were phase modulation experiments [4,5].

An accurate response function is important to extract physically correct dynamics. In practice the instrumental response function (IRF) is obtained by replacing the sample by a scattering medium, and recording the Rayleigh scattering of excitation light. In hitherto reported works on time-resolved multiphoton spectroscopy, this approach is also used. However, Rayleigh scattering is a linear process and consequently the response function obtained will not reflect the *true* response function in an MPE experiment. Furthermore, the femtosecond lasers used in time-resolved MPE operate at a high repetition rate that is usually reduced by using the cavity dumper technique. In practice, the extinction efficiency of cavity dumpers is limited and leakage pulses are produced. These pulses are not observed upon MPE, because of the quadratic dependence on the laser power. These extra pulses may limit the use of deconvolution methods in evaluating experimental decays over the whole time range. It is especially important for certain ranges of lifetimes and rotational correlation times. Present work solves these problems for 2-photon excitation (TPE) by making use of hyper Rayleigh scattering (HRS) of colloidal gold particles, previously observed by Clay *et al.*[6]. We here demonstrate that the response function recorded as HRS provides a substantial improvement in analyses of TPE fluorescence monitored by the TCSPC technique.

In the present work the excitation source for the 2-photon experiment was a 200-KHz, 3-μJ femtosecond laser amplifier system (Coherent radiation). The system consists of an Argon ion pumped Ti:sapphire mode locked laser (Mira), which is used to seed a Ti:sapphire regenerative laser amplifier (RegA 9000). The operating wave-

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length was 800 nm with fwhm pulses of 120 fs. For the 1-photon experiment, the 800-nm output was frequency doubled by directing the beam into the Coherent 9400 parametric amplifier. Further technical details concerning the experimental system and analyses of TCSPC data are given elsewhere [7]. Fluorescence was measured using standard fused silica cuvettes (10×10 m*M*). To minimize artefacts resulting from time-spread across the excitation beam, second-harmonics generated at and within the walls of the cuvette, scattered excitation light, and interference of possible emission from the cuvette walls some spatial masking was designed. The cuvette was masked with a 2-m*M* diameter aperture on the collection side in order to confine the observation to region of maximum 2-photon–induced fluorescence. The fluorescence emission was passed through appropriate band pass and cut-off filters to exclude excitation and second harmonic light. The instrument response function (IRF) was measured by replacing the sample with a scattering dispersion of colloidal gold particles. Fluorescence lifetime measurements were carried out under "magic angle" conditions. POPOP {1,4-di-2-(5-phenyl-oxoaconlyl)-benzene} was purchased from BDH Chemicals, Pole, England and used after recrystallization.

The colloidal gold particles were prepared using a method reported in the literature [8]. A particle size of 16 nm was determined by means of a transmission electron microscope.

Synthetic data mimicking time-correlated single-photon counting (TCSPC) experiments were generated using a Monte Carlo convolution method [9]. The true or theoretical decay curves ($I(t)$) of the fluorescence relaxation were convoluted with the experimental TP-IRF ($E(t)$) to form the observed intensities ($F(t)$) according to:

$$F(t) = \int_0^t E(t - t')\, I(t')dt' \qquad (1)$$

For the present case, poissonian noise was imposed on the $I(t)$ curves to resemble experimental data, while the experimental $E(t)$ was used as recorded. To minimize the inherent round-off errors of the TCSPC technique, each channel was split into 32 subchannels that were summed up after the convolution. To compare with true lifetime-experiments, the decay curves were generated with at least 10,000 counts in the peak channel.

The 1- and 2-photon (i. e., $n = 1$ and 2) excited fluorescence intensity ($F^{(n)}$) is proportional to the number density of molecules and the excitation photon flux density

$$F^{(n)} \propto \rho_{mol}\, \rho_{exc}^{n}\, \sigma^{(n)} \qquad (2)$$

In Eq. (2), ρ_{mol} and ρ_{exc}^{n} denote the number density of molecules (ρ_{mol}) and the excitation photon flux density (ρ_{exc}^{n}). In Eq. (1) the 1- and 2-photon absorption cross sections are denoted $\sigma^{(1)}$ and $\sigma^{(2)}$, respectively.

The OP and TP response functions were determined from measurements of Rayleigh and hyper Rayleigh scattering by colloidal gold particles at 800 nm and 400 nm, respectively. The quadratic dependence of the HRS on the laser intensity was used to confirm the non-linear nature of the scattered signal at 400 nm. Both response functions were recorded using the TP excitation wavelength (800 nm). There is a significant difference in the pulse shape as is seen in Fig. 1A. This is not compatible with the known wavelength/time dependence of multichannel plate detectors, which would only introduce a time shift of the pulse [10]. The TP-IRF clearly exhibits a faster rise time and a narrower pulse width. Although the fwhm of the pulses are about 120 fs, the fwhm of the IRF are typically about 1000 times longer. Hence the width of the IRF is dictated by the detection system.

The fluorescence intensity of POPOP in ethanol was studied as a function of the excitation intensity, which is directly proportional to the excitation photon flux density (ρ_{exc}) in Eq. (2), For TPE fluorescence, a log-log plot of the fluorescence signal vs. excitation intensity [cf. Eq. (2)] would yield that $n \approx 2$. Indeed, quadratic intensity dependence was also confirmed. The TCSPC decay of TPE fluorescence from POPOP in ethanol excited at 800 nm is shown in Fig. 1B and C. The data in panels B and C also display the result obtained by deconvolution using OP- and TP-IRF that were monitored as RS and HRS, respectively. The weighted residual plot clearly reveals that TP-IRF provides the best fit with experimental data. The results are summarized in Table I. Because of low absorptivity of POPOP at 400 nm, OPE is weak, implying that an influence from fluorescent impurities easily becomes noticeable. Indeed, this is the case; both Ti:sapphire and diode laser excitation at 400 nm reveal, in addition to the dominant lifetime component of POPOP ($\approx$1.3 ns), a weak contribution from a more long-lived component ($\approx$4.5 ns) [7]. The latter is compatible with presence of a fluorescent impurity. For TPE we also find bi-exponential fluorescence decays with lifetimes of about 1.3 and 4.5 ns. Despite this inconvenience, we compare deconvolutions of the TPE fluorescence decay when using the IRF recorded by OP and TP scattering, respectively. Taken together, using the TP-IRF is superior for the analyses of TPE fluorescence (cf. Table I).

To further explore the influence of using OP- and TP-IRF, fluorescence decay curves were generated for different single exponential decays of the fluorescence relaxation. Experimental data were then mimicked by

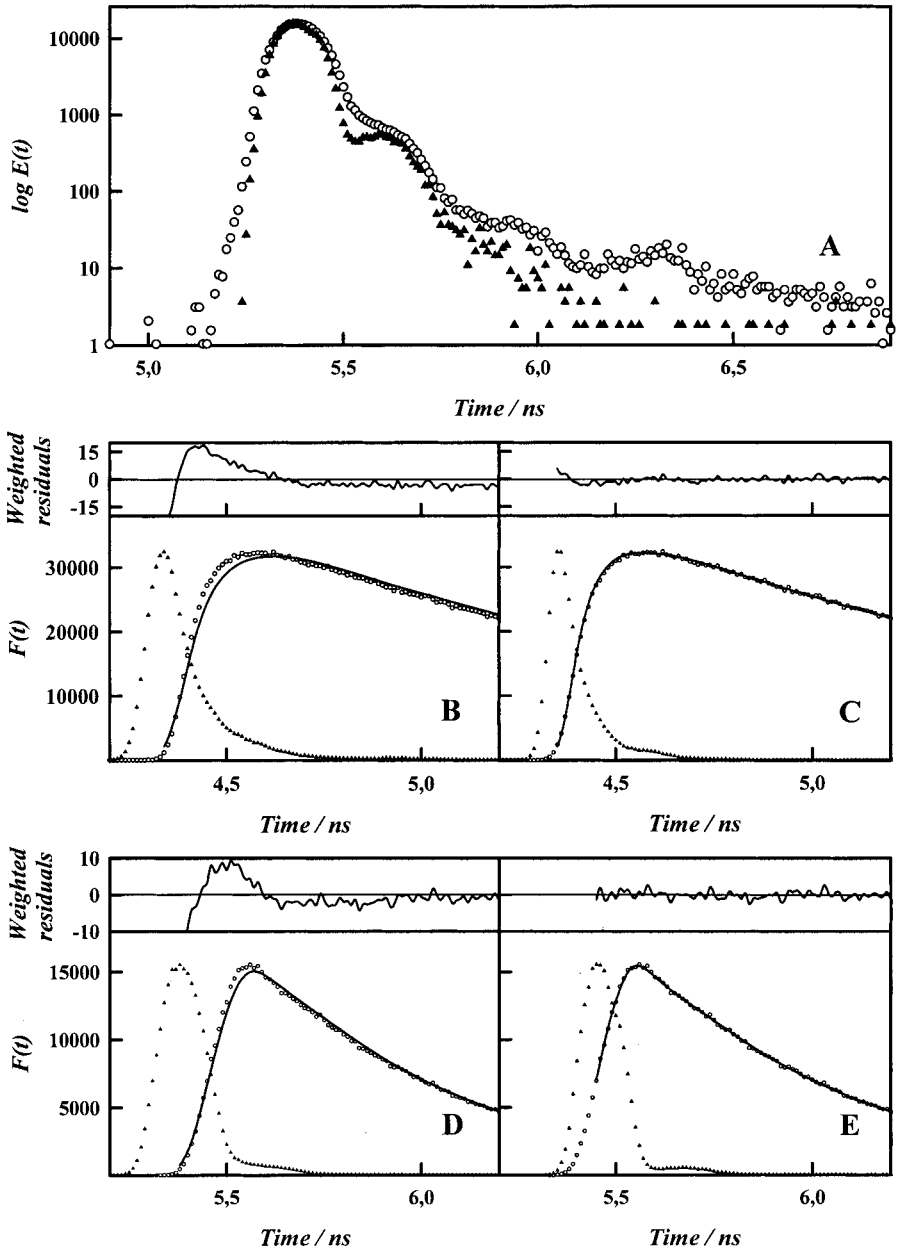

Fig. 1. The instrumental response functions obtained as Rayleigh scattering (RS, ○) and hyper Rayleigh scattering (HRS, ▲) for a system of colloidal gold particles (A). The fluorescence relaxation of POPOP in ethanol at 301 K analyzed by using the instrumental response function resulting from RS (B) and from HRS (C). The upper panels display the residual graphs for the cases of B and C. The mimicked TCSPC decay of a TP-excited compound that exhibits a single fluorescence lifetime (τ = 0.500 ns) was deconvoluted by using the instrumental response function resulting from RS (D) and HRS (E). Both functions were recorded for a system of colloidal gold particles. The upper panels display the residual graphs for the cases of D and E.

using an experimental TP-IRF for the lifetimes τ = 0.100, 0.200, 0.50, and 1 ns. To exemplify generated TCSPC data, the decay corresponding to a lifetime of τ = 0.500 ns is displayed in Fig. 1D and E. The results obtained by deconvolution of synthetic data using the OP- and TP-IRF are summarized in Table I. It is obvious that the deconvolutions using the OP-IRF in general are statistically worse. Hence, by using the OP-IRF, one might

Table I. Generated and Experimental Time-Correlated Single-Photon Counting Data with Fluorescence Lifetimes Varying from 0.1 ns to 1.32 ns, were Deconvoluted Using Both the One-Photon (OP-IRF) and the 2-Photon (TP-IRF) Instrumental Response Functions. The True or Expected Fluorescence Lifetime is Denoted τ, and the Deviation Between the Expected Lifetime and the Experimental (τ_{exp}) was Calculated According to $Error = \frac{\tau - \tau_{exp}}{\tau}$ 100%. The Statistical Parameter χ^2 and the Durbin-Wattson (D.-W.) Parameter Were Used to Judge the Quality of the Fit.

Response function	System	τ/ns	Error (%)	χ^2	D.-W.
OP-IRF	Synthetic	0.10	12.0	50.5	0.12
	Synthetic	0.20	6.5	15.2	0.24
	Synthetic	0.50	3.0	3.91	0.58
	Synthetic	1.00	1.9	2.20	0.92
	POPOP in ethanol	1.32[a]	3.7[b]	5.36	0.44
TP-IRF	Synthetic	0.10	0.0	1.15	1.94
	Synthetic	0.20	0.5	1.14	1.89
	Synthetic	0.50	0.4	1.04	1.96
	Synthetic	1.00	0.5	0.99	1.96
	POPOP in ethanol	1.32[a]	−3.0[b]	1.30	1.63

[a] From work by Kolber and Barkley [11]

[b] Fluorescence decays of POPOP were not single exponential functions. *Cf.* RESULTS AND DISCUSSION, as well as the work by Habenicht *et al*. [7]. A bi-exponential fit was applied and the *Error* was calculated using the shorter lifetime extracted.

erroneously conclude that the fluorescence relaxation is more complex than is actually the case.

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