Fluorescence Lifetime Standards for Time and Frequency Domain Fluorescence Spectroscopy

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A series of fluorophores with single-exponential fluorescence decays in liquid solution at 20 °C were measured independently by nine laboratories using single-photon timing and multifrequency phase and modulation fluorometry instruments with lasers as excitation source. The dyes that can serve as fluorescence lifetime standards for time-domain and frequency-domain measurements are all commercially available, are photostable under the conditions of the measurements, and are soluble in solvents of spectroscopic quality (methanol, cyclohexane, water). These lifetime standards are anthracene, 9-cyanoanthracene, 9,10-diphenylanthracene, *N*-methylcarbazole, coumarin 153, erythrosin B, *N*-acetyl-L-tryptophanamide, 1,4-bis(5-phenyloxazol-2-yl)benzene, 2,5-diphenyloxazole, rhodamine B, rubrene, *N*-(3-sulfopropyl)acridinium, and 1,4-diphenylbenzene. At 20 °C, the fluorescence lifetimes vary from 89 ps to 31.2 ns, depending on fluorescent dye and solvent, which is a useful range for modern pico- and nanosecond time-domain or mega- to gigahertz frequency-domain instrumentation. The decay times are independent of the excitation and emission wavelengths. Depend-

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ent on the structure of the dye and the solvent, the excitation wavelengths used range from 284 to 575 nm, the emission from 330 to 630 nm. These lifetime standards may be used to either calibrate or test the resolution of time- and frequency-domain instrumentation or as reference compounds to eliminate the color effect in photomultiplier tubes. Statistical analyses by means of two-sample charts indicate that there is no laboratory bias in the lifetime determinations. Moreover, statistical tests show that there is an excellent correlation between the lifetimes estimated by the time-domain and frequency-domain fluorometries. Comprehensive tables compiling the results for 20 (fluorescence lifetime standard/solvent) combinations are given.

Fluorescence decay measurements are an exceptionally useful tool for investigating the dynamics of excited states in biology, chemistry, and physics. The two main methods for obtaining time-resolved fluorescence data are single-photon timing (also called time-correlated single-photon counting) and multifrequency phase-modulation fluorometry. Both techniques yield essentially the same information and differ mainly in how the time-resolved fluorescence data are obtained, i.e., time domain (TD) versus frequency domain (FD). For detailed information on the two methods (general principles, instrumentation, data analysis, and possible problems with data collection and analysis), we refer to several excellent books and reviews.[1−13]

Fluorescence lifetime standards are needed most in the areas of photophysics, photobiology, chemical sensing, physical chemistry, fluorescence lifetime imaging microscopy, flow cytometry, and single-molecule spectroscopy. Fluorophores with known lifetimes are necessary for testing the time-resolved instruments for systematic errors, for calibration of fluorescence lifetime instruments, and for use as reference compounds to avoid the wavelength-dependent time−response (the color effect) of photomultiplier tubes.[14,15] Although these wavelength-dependent effects are less pronounced with microchannel plate photodetec‑

tors,[16−20] it is still necessary to verify that such effects are not present or to correct for them by the use of fluorescence lifetime standards.[13]

One of the earliest sources of single-exponential lifetime data is Birks's *Photophysics of Aromatic Molecules*,[21] published in 1970. However, many of the lifetimes compiled in this pioneering work have since been shown to be seriously in error. At the time the book was written, relatively crude techniques for lifetime measurements were in operation. Another early standard work for lifetime data is Berlman's *Handbook of Fluorescence Spectra of Aromatic Molecules*.[22] Because these lifetimes were obtained more than 40 years ago with an old-fashioned pulse sampling oscilloscope technique, it should not come as a surprise that many of them were found to be inaccurate. In 1974, Chen[23] proposed quinine and γ-pyrenebutyrate for use as lifetime standards. Later research[24] showed that quinine is unsuitable because it exhibits dual-exponential decay kinetics, whereas γ-pyrenebutyrate is easily photolyzed and sensitive to quenching by oxygen. A few years later, Grinvald[25] proposed *N*-acetyl-L-tryptophanamide, anthracene, and naphthalene as lifetime standards. While the first two may be useful standards, the long lifetime of naphthalene is very sensitive to oxygen quenching and makes naphthalene unsuitable as lifetime standard (see Lifetimes). The availability since 1978 of high repetition rate, mode-locked, sync-pumped (cavity-dumped) dye lasers[26,27] as picosecond excitation sources in TD and FD fluorometry allowed the very rapid collection of time-resolved fluorescence data and stimulated the search for more reliable fluorescence lifetime standards. Several groups contributing to the current study embarked separately on projects aimed at obtaining accurate nanosecond[11,28,29] and picosecond[11,20,30] lifetime values. Unfortunately, only a limited number of the same lifetime standards have been measured independently by different laboratories so that the reliability of the reported lifetime data is unclear. Moreover, no attempt has been made to compare the lifetime values or to evaluate which values are more reliable. Finally, until now, no systematic comparison has been carried out of the precision of the lifetime data obtained by the TD and FD methods.

The conclusions of the earlier studies on fluorescence lifetime standards can be summarized as follows. There are only a few (fluorescent lifetime standard/solvent) combinations reported in

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the literature that have been measured by several laboratories with sufficiently high precision (Tables S8 and S9, Supporting Information) and some of the proposed ones, like quinine bisulfate, are unsuitable because they do not display single-exponential decays.[24] Furthermore, many of the proposed substances have lifetimes that are too long to provide a meaningful test for picosecond instrumentation. Additionally, long lifetime standards are difficult to use due to the effect of oxygen quenching. The use of collisional quenching to obtain different lifetimes[23] is not advocated for lifetime standards, because of the risk of transient effects and nonexponential decays. Finally, the compounds cited in the literature do not cover fully the spectral region of interest (both in excitation and emission).

To provide the research community with reliable fluorescence lifetime standards, an international, cooperative project, which involved nine research groups active in the field of time-resolved fluorescence, was initiated by the corresponding authors. Those nine laboratories from the United States, Canada, the U.K., Japan, and Continental Europe are identified in the text and the Supporting Information by a three-letter label based on the university or the associated university city (BAL = Baltimore, HAS = Hasselt, IRV = Irvine, LEU = Leuven, LON = London, NIS = Nishinomiya, PAR = Paris, WAG = Wageningen, WLU = Wilfrid Laurier University).

For the venture to be successful, the following preconditions were set. Because a reliable lifetime value for a standard should be independent of the used measurement method, research groups using the TD and FD methods were invited to join the project. The combination of groups active in TD and FD allows one to verify the equivalence of the precision of both methods. Another prerequisite was the use of lasers as excitation sources. The majority of the instruments was equipped with microchannel plate photodetectors. This combination produces greater excitation light intensity and improved temporal resolution when compared to older setups. All phase and modulation measurements were done at multiple modulation frequencies.

CRITERIA FOR THE CHOICE OF FLUORESCENCE LIFETIME STANDARDS AND SOLVENTS

Although any compound with a single-exponential decay can theoretically serve as a lifetime standard, for the sake of convenience and standardization, the following criteria for the choice of possible lifetime standards in liquid solution were applied. (i) A first condition for a lifetime standard is that it should show single-exponential decay kinetics, independent of excitation and emission wavelength. (ii) The compound should be commercially available in sufficiently high purity (e.g., laser grade, scintillation grade, zone-refined, ≥99%, etc.) so that additional purification steps can be avoided. Our original aim was to evade dyes that have to be purified prior to the measurements, because the necessary skills/equipment to purify a compound to fluorometric grade might not always be on hand. Unfortunately, some of the commercial compounds, which were candidates for lifetime standards, showed dual-exponential fluorescence decay kinetics when used as received (see Table S4, Supporting Information). So, we had to relax this criterion for some compounds because the existing purity of the commercial products was not high enough. Therefore, some of the compounds were purified before the fluorescence lifetime measurements. (iii) From a practical point of view, an ideal

fluorescence lifetime standard should have a (relatively) large Stokes shift (to ensure minimal spectral overlap of excitation and emission spectra) and a (relatively) large quantum yield. (iv) To cover the picosecond and nanosecond time scales, evidently a series of fluorescence standards with lifetimes matching that range should be on hand. (v) A variety of fluorescence standards should be available to cover different spectral regions. (vi) Insofar as possible, the standards should not pose health, safety, or environmental problems. (vii) A final criterion is chemical stability and photostability during the fluorescence measurements.

The choice of solvents was guided by their commercial availability for fluorescence measurements. Water is an environment benign solvent. Ultrahigh quality water, delivered by a properly maintained Milli-Q system (Millipore),[31] meets all the requirements of fluorescence spectroscopy. The life science and biotechnological communities prefer fluorescent standards in aqueous solution. Methanol, cyclohexane, and methylcyclohexane can be obtained in sufficiently high (spectroscopic) purity from several chemical suppliers. Good laboratory practices should be followed during storage, usage, and disposal of the lifetime standards and solvents.

THEORY

Kinetics. For a fluorophore that decays monoexponentially with lifetime τ, the fluorescence δ-response function $f(t)$ is

$$f(t) = \alpha \exp(-t/\tau) \tag{1}$$

where α is the pre-exponential factor or amplitude.

In single-photon timing experiments where the fluorophore is excited by an excitation pulse $u(t)$, the observed fluorescence decay, $y(t)$, is a convolution of $f(t)$ and $u(t)$:

$$y(t) = \int_0^t f(t')u(t - t')\, \mathrm{d}t' = \int_0^t f(t - t')u(t')\, \mathrm{d}t' = f \otimes u \tag{2}$$

where $\otimes$ denotes the convolution operator and t and t' represent time. The instrument response function $u(t)$ is usually obtained by measuring the scattered excitation light. When $y(t)$ and $u(t)$ are experimentally known, the parameters α and τ of $f(t)$ can be estimated by a variety of techniques.[32−36]

In the frequency domain, the values of the phase shift, ϕ, and the relative modulation, m, for a fluorophore with lifetime τ, are given by

$$\phi = \tan^{-1}(\omega\tau) \tag{3}$$

$$m = \frac{1}{\sqrt{1 + \omega^2\tau^2}} \tag{4}$$

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where $\omega = 2\pi f$ is the angular frequency of the harmonically modulated excitation light and f the generator-set frequency (expressed in Hz). The lifetime can thus be independently determined from the phase shift (eq 3) and the relative modulation (eq 4). The lifetimes determined from phase and modulation data are equal regardless of the modulation frequency of the excitation light for a single-exponential decay while this is not the case for a multiexponential decay.

To correct for the wavelength dependence of the instrument response function $u(t)$ (sometimes referred to as color effect) in single-photon timing experiments, the use of the reference convolution method is recommended.[37−42] This method (also called delta function convolution method[39] or F/F deconvolution[38]) requires a single-exponential reference, which has to be measured under identical instrumental settings as used for the sample. Under these conditions, the parameters of the modified sample decay, $\tilde{f}(t)$, are obtained from the measured decays of the sample, $y(t)$, and reference, $d_r(t)$:

$$y(t) = \int_0^t \tilde{f}(t')d_r(t-t')\ \mathrm{d}t' = \int_0^t \tilde{f}(t-t')d_r(t')\ \mathrm{d}t' = \tilde{f} \otimes d_r \tag{5}$$

When $f(t)$ is single-exponential, $\tilde{f}(t)$ is given by eq 6, where $\delta(t)$ denotes the Dirac δ-function and τ_r the reference lifetime:

$$\tilde{f}(t) = \alpha[\delta(t) + (1/\tau_r - 1/\tau)\exp(-t/\tau)] \tag{6}$$

A variety of analysis methods[32] is accessible to estimate α, τ, and τ_r of $\tilde{f}(t)$ once $y(t)$ and $d_r(t)$ are known.

To correct for the photomultiplier tube color effect in FD measurements, the phase shift and relative modulation are measured under identical instrumental settings for a reference fluorophore with lifetime τ_r and the sample fluorophore. The values for the phase difference $\Delta\phi$ and modulation ratio m/m_r between two fluorophores that decay monoexponentially are given by[29]

$$\Delta\phi = \tan^{-1}(\omega\tau) - \tan^{-1}(\omega\tau_r) \tag{7}$$

$$\frac{m}{m_r} = \sqrt{\frac{1 + \omega^2\tau_r^2}{1 + \omega^2\tau^2}} \tag{8}$$

where m_r denotes the modulation of the reference.

Single-Curve Fitting. (1) Data Analysis. In TD experiments, the nonlinear mathematical model is of the form $y = f(t,\theta)$ where t (time) and y (decay data) are observable variables and θ is the unknown parameter vector with p components. To estimate θ, one observes y at n values of t. In FD measurements, the nonlinear model is of the form $y = f(\omega,\theta)$ for phase and $z = g(\omega,\theta)$ for

modulation, where ω (angular frequency of light modulation) is an observable variable. To estimate θ, one observes y and z at n values of ω. (Note that the number n of modulation frequencies ω used in FD measurements is generally smaller than the number n of used values of t in the TD method.) Then $\hat{\theta}$, the weighted least-squares estimate of θ, is determined by minimizing the reduced chi-square value χ_ν^2:

$$\chi_\nu^2 = \sum_i^n w_i(y_i^o - y_i^c)^2/\nu = \sum_i^n R_i^2/\nu \tag{9}$$

In eq 9, the index i sums over n data points, y_i^o and y_i^c denote respectively the $o$bserved (experimental) and $c$alculated (fitted) values corresponding to the ith data point, and $w_i = 1/\sigma_i^2$ denotes the associated statistical weight, where σ_i^2 is the variance of y_i^o. $R_i = (y_i^o - y_i^c)/\sigma_i$ stands for the weighted residual of the ith data point; $\nu = n - p$ is the number of degrees of freedom with p the number of adjustable fit parameters.

(2) Statistical Criteria for the Quality of the Fit. A characteristic feature of single-photon timing experiments is the statistical nature of decay data accumulation. In the absence of systematic errors, the number of counts in each channel follows a Poisson distribution, so that in the Gaussian limit $\sigma_i^2 = y_i^o$. For FD fluorometry, one has to determine the variances σ_ϕ^2 and σ_m^2 of ϕ and m, respectively, by repeated measurements at each ω. Since the variance of each data point, measured by the single-photon timing technique (TD), is known, a rigorous statistical assessment of acceptability of fits is possible. Conversely, the variances of phase shift ϕ and relative modulation m calculated from many FD measurements at a given modulation frequency ω are often underestimated (because of systematic errors), as shown by the abnormally high χ_ν^2 values obtained when using these values. The comparison of the χ_ν^2 values for different models is a better test for model discrimination (e.g., dual-exponential decay versus single-exponential decay) than the absolute χ_ν^2 value.

A rigorous examination of the deviations between the experimental and fitted data comprises both graphical techniques (weighted residuals, the autocorrelation function,[43] and the normal probability plot of the weighted residuals[44,45]) and numerical statistical tests (χ_ν^2 and its standard normal deviate $Z\chi_\nu^2$, the Durbin−Watson test statistic,[46] and the ordinary runs test[45,47]). Detailed descriptions of the various tests to assess the quality of the fit in single-curve analysis are given in the Supporting Information.

Global Analysis. The advantages of the global (or simultaneous) analysis method[48−52] in comparison to single-curve analysis

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are improved model testing capability and accuracy of the estimated parameters.[53]

(1) Data Analysis. Instead of minimizing χ_ν^2 defined in eq 9, in global analysis one minimizes the global $\chi_{\nu g}^2$,

$$\chi_{\nu g}^2 = \{\sum_l^q \sum_i w_i(y_i^o - y_i^c)^2/\nu_g\} \qquad (10)$$

where the subscript g denotes global. The index l sums over q experiments, whereas the index i sums over the appropriate number of data points for each individual experiment. ν_g represents the number of degrees of freedom for the entire multidimensional fluorescence decay surface. Equation 10 is formally equivalent to eq 9, but the summation is over the entire decay surface.

(2) Statistical Criteria for the Quality of the Fit. The graphical methods include plots of surfaces (carpets) of the autocorrelation function values[43] versus experiment number and of the weighted residuals versus time (or channel number, TD) or frequency ω (FD) versus experiment number. A good fit should produce carpets free of creases. The numerical statistical tests are the calculation of the global $\chi_{\nu g}^2$ (eq 10) and its corresponding $Z\chi_{\nu g}^2$

$$Z\chi_{\nu g}^2 = \sqrt{\nu_g/2}(\chi_{\nu g}^2 - 1) \qquad (11)$$

Since $Z\chi_{\nu g}^2$ is standard normally distributed, $Z\chi_{\nu g}^2$ can be readily used to compare the goodness of fit of analyses with different ν_g. The goodness of fit for the individual decay curves (TD) or phase and modulation data (FD) is examined by the Durbin–Watson statistic,[46] the ordinary runs test,[45,47] the local χ_ν^2 (eq 9) and its normal deviate $Z\chi_\nu^2$.

EXPERIMENTAL SECTION

Materials. All fluorophores [anthracene, 9-cyanoanthracene, 9,10-diphenylanthracene (DPA), N-methylcarbazole, coumarin 153, erythrosin B, N-acetyl-L-tryptophanamide (NATA), 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP), 2,5-diphenyloxazole (PPO), rhodamine B, rubrene, N-(3-sulfopropyl)acridinium (SPA), 1,4-diphenylbenzene (p-terphenyl)] were typically of the highest purity commercially obtainable (e.g., laser grade, 99%, scintillation grade, zone-refined, gold label, microscopic grade, etc.) and were procured from different commercial chemical suppliers. It is not the purpose of this report to distinguish "good" from "bad" suppliers of fluorescent dyes or to name the "best" buy for a specific dye. Indeed, the quality of a certain dye procured from a particular provider might change over time or from lot to lot. It also is possible that chemical suppliers will merge with other companies or cease to exist. *Our aim is to determine if a certain fluorescent dye displays single-exponential decays and can be used as a robust lifetime standard.*

The solid samples of the fluorescence lifetime standards are stable when stored according to the supplier's instructions. The solvents (methanol, cyclohexane, methylcyclohexane) were of spectrophotometric grade, HPLC grade, fluorometric grade, high purity, etc., and were obtained from different chemical suppliers. The solvents showed negligible background fluorescence under the spectral conditions of the experiments. When water was used as solvent, it was of Milli-Q (Millipore)[31] quality or doubly distilled. Some measurements were done in ACS certified aqueous buffer pH 7 in lieu of pure water.

Instrumentation. Multifrequency phase and modulation measurements were performed using lasers as excitation source, either mode-locked lasers (IRV, LEU, BAL) or cw lasers associated with a Pockels cell (PAR), as previously described by the different research groups participating in the current study.[7] Between 11 and 60 frequencies f (25 is the average) were used in the measurements. The number of frequencies that is required to recover "correct" values for the decay parameters depends on the complexity of the model decay function $f(t)$. In principle, a few frequencies suffice for a single-exponential whereas at least 50 or more frequencies (producing ≥ 50 ϕ and ≥ 50 m values) are necessary for a distribution of exponentials. Laser excitation was similarly used in the single-photon timing measurements, as described in the literature.[54] The decay traces were collected in the channels (between 1/4K and 4K) of a (computer-integrated) multichannel analyzer. Since the obtained results are independent of the used FD and TD instrumentation (see Laboratory Bias and Comparison of Precision of TD and FD Methods), detailed instrumental descriptions will not be given here and can be found in the literature. The used excitation and emission wavelength ranges are compiled in Table 1. The excitation wavelength ranges might not be the optimal ones (see Figure S1, Supporting Information), because the best possible laser excitation wavelengths were not accessible on some laser setups. The absorbance of the fluorophores in all solutions at the excitation wavelength was less than 0.15, typically ~0.05. Magic angle (54°44′) detection[55] was used to eliminate the effects of rotational diffusion on the intensity decays.[11–13] If that preventative measure is not taken, the measured time-resolved fluorescence trace will be at least biexponential (eq 15 in Lifetimes; see the results of LON in Table S4, Supporting Information). All TD laboratories as well as LEU (FD) used a monochromator to select the emission wavelength. The FD laboratories IRV, PAR, and in some instances BAL used optical filters for this purpose.

Dissolved oxygen was removed from all solutions by repetitive freeze–pump–thaw cycles or by purging the solutions with N_2 or Ar. The steady-state intensities of the samples were stable during the lifetime measurements. Excitation and emission spectra were recorded on a SPEX Fluorolog fluorometer and are corrected for fluctuations of the excitation source flux and wavelength dependence of the detection system. Excitation spectra closely matched absorption spectra, taken on a Perkin-Elmer Lambda 40 UV–visible spectrophotometer. All steady-state and time-resolved measurements were done at 20 °C. Figure S1 (Supporting Information) displays the normalized absorption and emission

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Table 1. Mean Lifetime Data $\bar{\tau}$ of the Fluorescent Lifetime Reference Dyes in Fluid Solution at 20 °C Measured in the Present Study

compound[a]	solvent	lifetime $\bar{\tau} \pm s$ (ns)[b]	$100\,s/\bar{\tau}$	λ_{ex} (nm)	λ_{em} (nm)	c	n[d]
anthracene	methanol	5.1 ± 0.3	6.1	295−360	375−442	7	7
	cyclohexane	5.3 ± 0.1	2.6	295−360	375−442	7	7
9-cyanoanthracene	methanol	16 ± 1	9.3	295−360	400−480	7	7
	cyclohexane	12.7 ± 0.7	5.5	295−360	400−450	4	4
DPA	methanol	8.7 ± 0.5	5.6	295−360	400−475	8	8
	cyclohexane	7.5 ± 0.4	5.8	295−360	400−475	8	**7**
N-methylcarbazole	cyclohexane	14.1 ± 0.9	6.2	290−325	350−400	6	6
coumarin 153	methanol	4.3 ± 0.2	4.5	295−442	495−550	5	5
erythrosin B	water	0.089 ± 0.003	3.6	488−568	550−580	6	6
	methanol	0.47 ± 0.02	4.0	488−568	550−590	6	6
NATA	water	3.1 ± 0.1	3.6	295−309	330−410	7	7
POPOP	cyclohexane	1.12 ± 0.04	3.6	295−360	380−450	8	8
PPO	methanol	1.65 ± 0.05	2.7	295−330	340−400	8	8
	cyclohexane	1.36 ± 0.04	2.6	290−325	360−450	8	8
rhodamine B	water	1.74 ± 0.02	0.9	488−575	560−630	6	**5**
	methanol	2.5 ± 0.1	4.0	295, 488−568	550−630	8	8
rubrene	methanol	9.9 ± 0.3	3.2	300,488,514	550−610	5	5
SPA	water	31.2 ± 0.4	1.4	300−330	466−520	5	5
p-terphenyl	methanol	1.17 ± 0.08	6.5	284−315	330−380	7	7
	cyclohexane	0.98 ± 0.03	3.3	290−315	330−390	7	7

[a] For abreviations used, see text. All solutions are deoxygenated by repetitive freeze−pump−thaw cycles or by bubbling N_2 or Ar through the sample solutions. [b] Average lifetime $\bar{\tau}$. The quoted errors are sample standard deviations[64] $s = \lfloor (n-1) \sum_{n=1}^{n} (\tau_i - \bar{\tau})^2 \rfloor^{1/2}$. [c] Number of lifetime data measured. [d] Number of lifetime data used in the calculation of the mean lifetime $\bar{\tau}$ and its standard deviation[64] s. The difference between columns c and d gives the number of outliers. Boldface numbers in column d indicate that there is one outlier present.

spectra of all (fluorescence lifetime standard/solvent) combinations measured in this work. Additionally, this figure gives the chemical formula, the formula weight, the CAS number, and the fluorescence quantum yield of each of the fluorescent lifetime standards.

Data Analysis. Each research group measured and analyzed its own time-resolved fluorescence data, using its individual data analysis software. To minimize any bias in the lifetime data, the fluorescence lifetime values obtained by the different laboratories were hidden from each other until the final version of this paper was distributed to the participating laboratories for final editing. Only the corresponding authors had prior access to all the data (for the statistical analysis). Since the used curve-fitting programs are not a critical factor, we shall not describe them here; details can be found in the literature.[41,56,57] LON analyzed the data as outlined in ref 3. The criteria to evaluate the quality of the fits are described in Single-Curve Fitting and Global Analysis and in the Supporting Information. The number and type of goodness-of-fit criteria varied among the contributing laboratories. Two FD laboratories (IRV, LEU) collected their phase shift and modulation data versus reference fluorophores, while four laboratories (BAL, LON, NIS, WLU) recorded the phase shift/modulation data and fluorescence decays versus a Ludox or glycogen scattering solution in water. The remaining three research groups (LEU/HAS, PAR, WAG) used both reference fluorophores and a scattering solution. The lifetimes of sample (τ) and reference (τ_r) were modifiable fit parameters. In most cases, the fluorophores used as references were also the fluorescence lifetime standards

used in this study (Table 1). For example, erythrosin B in water as sample was measured versus erythrosin B in methanol as reference, and vice versa; PPO in cyclohexane as sample was measured versus *p*-terphenyl in cyclohexane, and vice versa; and so on.

RESULTS

Lifetimes. Table S4 (Supporting Information) shows all estimated lifetime values of 20 (fluorescent lifetime standard/solvent) combinations measured by the various laboratories. All time-resolved fluorescence data were analyzed by single-curve analysis. Three laboratories (LEU/HAS, LEU, LON) also used simultaneous (global) analysis of time-resolved fluorescence data measured at different emission wavelengths to verify the single-exponential character of the fluorescence decays. The values of the various criteria for assessing the quality of the fits indicated that all the decay data gave excellent single-exponential fits except the following.

9,10-Diphenylanthracene (97%), which was used as received from two different chemical suppliers, consistently yielded biexponential fits (eq 12).

$$f(t) = \alpha_1 \exp(-t/\tau_1) + \alpha_2 \exp(-t/\tau_2) \qquad (12)$$

It is only after recrystallization or sublimation that this compound displays single-exponential decay kinetics. One group (IRV) reported a biexponential decay for *N*-methylcarbazole (99%) in cyclohexane. The relative amplitude α_r [$= \alpha_1/(\alpha_1 + \alpha_2)$] of the 14.3-ns component was 0.902. Two biexponential fits were reported for coumarin 153 in methanol (out of five measurements). Apparently, laser grade quality from various suppliers leads to different (single-exponential versus dual-exponential) time-resolved

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Table 2. Comparison of FD and TD Data Precision of the Fluorescent Lifetime Reference Dyes in Fluid Solution at 20 °C Measured in the Present Study

compound[a]	solvent[b]	$\bar{\tau}_{FD}$ (ns)[c]	n_{FD}[d]	s_{FD}^{2}[e]	$\bar{\tau}_{TD}$ (ns)[c]	n_{TD}[d]	s_{TD}^{2}[e]	F[f]	F[g]	t[h]
anthracene	MeOH	5.00	3	0.191	5.20	4	0.042	4.54	16.04	0.819
	CyH	5.32	4	0.025	5.32	3	0.020	1.22	39.17	0.004
9-cyanoanthracene	MeOH	15.29	3	2.967	16.27	4	1.804	1.64	16.04	0.850
	CyH	12.39	3	0.294	13.47	1				
DPA	MeOH	8.71	3	0.541	8.77	5	0.141	3.83	10.65	0.152
	CyH	7.17	3	0.232	7.76	4	0.026	8.86	16.04	2.347
N-methylcarbazole	CyH	14.06	3	0.188	14.15	3	1.691	0.11	39.00	0.108
coumarin 153	MeOH	4.18	2	0.006	4.33	3	0.056	9.24	779	0.857
erythrosin B	water	0.090	2	1×10^{-5}	0.089	4	1×10^{-5}	1.02	17.44	0.247
	MeOH	0.45	2	2×10^{-5}	0.48	4	3×10^{-4}	18.16	864	1.772
NATA	water	3.14	3	0.02	3.01	4	0.003	4.82	16.04	1.925
POPOP	CyH	1.12	4	0.003	1.12	4	7×10^{-4}	4.22	15.44	0.016
PPO	MeOH	1.63	3	0.003	1.66	5	0.002	1.80	10.65	0.946
	CyH	1.35	4	0.002	1.38	4	6×10^{-4}	2.55	15.44	1.576
rhodamine B	water	1.73	1		1.75	4	0.000			
	MeOH	2.48	4	0.010	2.44	4	0.011	1.14	15.44	0.614
rubrene	MeOH	9.79	2	0.051	9.97	3	0.156	3.05	779	0.567
SPA	Water	30.90	2	0.076	31.37	3	0.185	2.43	779	1.359
p-terphenyl	MeOH	1.10	2	4×10^{-5}	1.20	5	0.005	122.91	899	1.913
	CyH	0.96	3	9×10^{-4}	1.00	4	1×10^{-4}	7.80	16.04	2.971

[a] Abbreviations used; see text. [b] MeOH = methanol, CyH = cyclohexane. [c] Average lifetime $\bar{\tau}_{FD}$ (respectively $\bar{\tau}_{TD}$) determined from FD (respectively TD) measurements. [d] Number of FD (TD) lifetime data used in the calculation of $\bar{\tau}_{FD}$ ($\bar{\tau}_{TD}$) and its corresponding variance s_{FD}^{2} (s_{TD}^{2}). [e] Variance for FD (respectively TD) measurements. [f] F-statistic calculated according to eq 16. [g] Tabled two-sided F-distribution value with α equal to 5%. This is equivalent to the cumulative function equal to 0.975. [h] Student's t-statistic calculated according to eq 17.

fluorescence. Five out of seven laboratories found clear monoexponential decay kinetics for NATA in water. The τ-value (3.28 ns) determined by BAL is a borderline value (see Outlier Detection). In some cases, NATA (98% and reagent grade) must be recrystallized to yield monoexponential decays, whereas in other cases, NATA (98% and reagent grade), procured from a different supplier, gave excellent single-exponential fits. Three groups out of six found biexponential decays for rhodamine B in water or aqueous buffer. The τ-value (2.8 ns) obtained by IRV is a clear outlier (see Outlier Detection). Rhodamine B in water readily forms aggregates (even at very low concentrations),[58–63] and therefore, one has to keep its concentration low enough for the time-resolved fluorescence to be single-exponential. For rhodamine B in methanol, excellent single-exponential fits were obtained by six out of eight groups. A single group (WAG) found a biexponential fit for rubrene in methanol. The relative amplitude α_r of the 9.58-ns component was 0.941. Two laboratories out of five reported biexponential fits for SPA in water. The relative amplitude α_r of the 31.09-ns component was 0.994 (PAR) and 0.917 for the 31.38-ns component (WAG). Apparently, SPA's purity varies between different lots (all SPA came from the same supplier) and SPA has to be purified to ensure excellent single-exponential fits.

At the start of the lifetime standards project, 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ), naphthalene, and L-tyrosine were also candidates as fluorescence lifetime standards. However, these

compounds cannot be recommended as lifetime references for different reasons. The six research groups that measured the time-resolved fluorescence of SPQ in water all reported biexponential fits. Naphthalene is not included in Tables 1 and 2 because the two available, estimated τ-values in methylcyclohexane vary too widely (95 and 113 ns). The value of 113 ns was obtained for a naphthalene solution degassed by four freeze–pump–thaw cycles and the 95-ns value for a solution deoxygenated by purging with N_2 gas. This may reflect the fact that naphthalene's long lifetime is still quenched by the residual oxygen left by the less efficient deoxygenation method. Furthermore, since the only decay of naphthalene measured in cyclohexane yielded a dual-exponential fit, naphthalene cannot be recommended as a lifetime standard. Because we only have three reliable lifetime data for L-tyrosine in water, we cannot endorse this amino acid as a lifetime standard, although the existing experimental evidence points to a single-exponential fluorescence decay (average lifetime $\bar{\tau}$ = 3.1 ns, sample standard deviation[64] s = 0.4 ns).

Even if the dye is ultrapure, its measured fluorescence decay cannot be described by a single-exponential function if the effect of the rotational motion is not eliminated by measuring under the magic angle condition. Indeed, the fluorescence light will in general be polarized to some extent depending on the geometry of the experimental setup and the rotational motion of the emitting fluorophore. Consider the case of a macroscopically isotropic sample excited by light polarized along the perpendicular to the

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excitation−detection plane and where the fluorescence is detected at 90° with respect to the direction of the excitation beam. The polarized fluorescence decays with the emission polarizer set parallel ($i_{\parallel}$) and perpendicular ($i_{\perp}$) to the polarization of the excitation light can then be written as[11,12]

$$i_{\parallel}(t) = \frac{1}{3} f(t) [1 + 2r(t)] \qquad (13a)$$

$$i_{\perp}(t) = \frac{1}{3} f(t) [1 - r(t)] \qquad (13b)$$

where $r(t)$ stands for the time-resolved fluorescence anisotropy. For an excited species in a single type of isotropic environment, $r(t)$ is, in general, given by a linear combination of exponentially decaying functions:

$$r(t) = \sum_j \beta_j \exp(-t/\phi_j) \qquad (14)$$

Only when the rotational relaxation times ϕ_j are much shorter than the fluorescence lifetime τ, the intensities $i_{\parallel}$ and $i_{\perp}$ become equal and proportional to $f(t)$. If this condition is not fulfilled, a polarizer oriented at 54°44′ with respect to the vertical has to be inserted in the emission path to eliminate the effect of rotational diffusion.[55] If the emission polarizer is absent, the fluorescence δ-response $i(t)$ can be written as

$$i(t) = \alpha_1 \exp(-t/\tau) + \alpha_2 \exp\left[-t\left(\frac{1}{\tau} + \frac{1}{\phi}\right)\right] \qquad (15)$$

for a single-exponential $r(t)$. For example, global biexponential analysis by LON of four decays collected at different emission wavelengths (420, 430, 440, 450 nm) for POPOP in cyclohexane ($\lambda_{ex} = 315$ nm) yielded a value of 1.11 ns for τ and 119 ps for the second decay time, $\tau\phi/(\tau + \phi)$. Similarly, biexponential decays were also obtained by LON for PPO in methanol and cyclohexane and for p-terphenyl in methanol and cyclohexane (Table S4, Supporting Information).

Table 1 summarizes the calculated mean lifetimes $\bar{\tau}$ and the associated sample standard deviations[64] s for each compound in the used solvents, based on the data of Table S4 (Supporting Information). Comparison of columns c and d of Table 1 indicates that two data points were deleted from the calculations, because they are not consonant with the rest of the data. Such outliers can only be removed from the data set after close examination, as described in Outlier Detection and the Supporting Information. Figure 1 shows graphically the lifetimes τ estimated by the different laboratories for two illustrative examples (POPOP in cyclohexane and DPA in cyclohexane). Figure 2 displays a typical fluorescence decay of PPO in cyclohexane obtained by the single-photon timing (TD) technique. The best fit, the instrument response function $u(t)$, the weighted residuals R_i, and the autocorrelation function C_j are also shown. Figure 3 shows the corresponding data for PPO in cyclohexane obtained by the phase-modulation (FD) technique. The best fits to the experimental phase shift ϕ and modulation data m, and the weighted residuals for both (ϕ, m) fits are also shown.
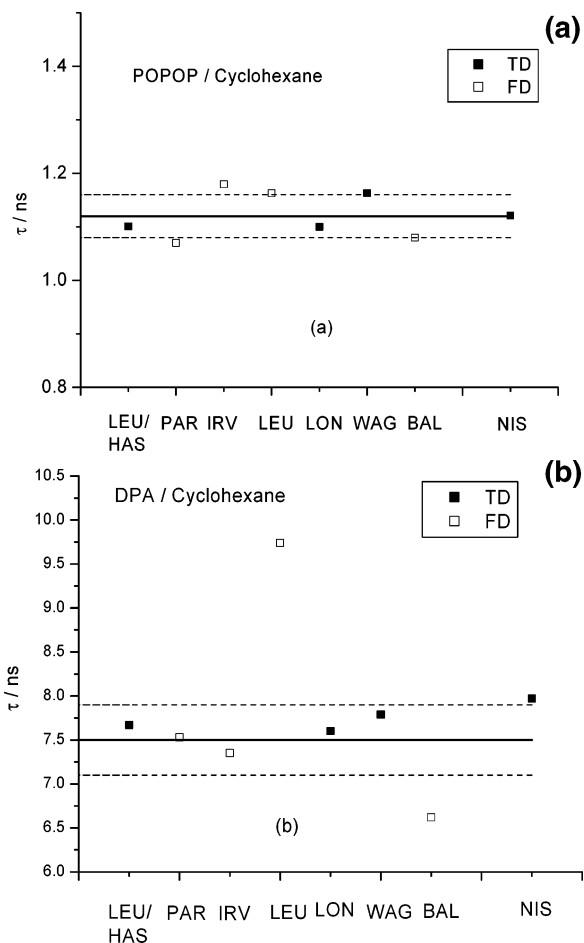
Figure 1. (a) Lifetimes τ of POPOP in cyclohexane at 20 °C estimated by the various laboratories. The boldface solid line indicates the average value $\bar{\tau}$; the dashed lines represent $\bar{\tau} \pm$ one sample standard deviation s (standard uncertainty u).[64] (b) Corresponding figure for DPA in cyclohexane. The point determined by LEU (FD) is an outlier and was not taken into account in the calculation of the average lifetime $\bar{\tau}$ and its standard deviation[64] s.

To have an idea of the precision of the measured lifetime data, we calculated the relative standard deviation (the ratio of the sample standard deviation[64] s over the mean lifetime $\bar{\tau}$) expressed as a percentage, i.e., $100\ s/\bar{\tau}$. These percentages range from 0.9% for rhodamine B in water to 9.3% for 9-cyanoanthracene in methanol (Table 1).

Outlier Detection. A potential outlier is an observation that has an extremely large deviation: a peculiarity that does not fit in with the pattern of the rest of the data points. Removing an outlying observation from the data set should be done only after close inspection of the data. There are two methods for detecting outliers: graphical and statistical.[45] For a discussion of the use of plotting techniques in outlier detection we refer to the literature.[45] The simplest technique is by examining a scatter plot of the original data (lifetime τ_i versus laboratory i as in Figures 1 and S2, Supporting Information) or, equivalently, of the (raw) residuals r_i ($r_i = \tau_i - \bar{\tau}$, with $\bar{\tau}$ mean lifetime) versus laboratory i. Extreme points are often easily visible on such graphs. Visual inspection of all 20 plots of τ_i versus laboratory i indicates that there are three possible outliers. Figure 1a (POPOP in cyclohexane) is indicative of the absence of outliers, while the data point from LEU (FD) for DPA in cyclohexane is a potential outlier (Figure
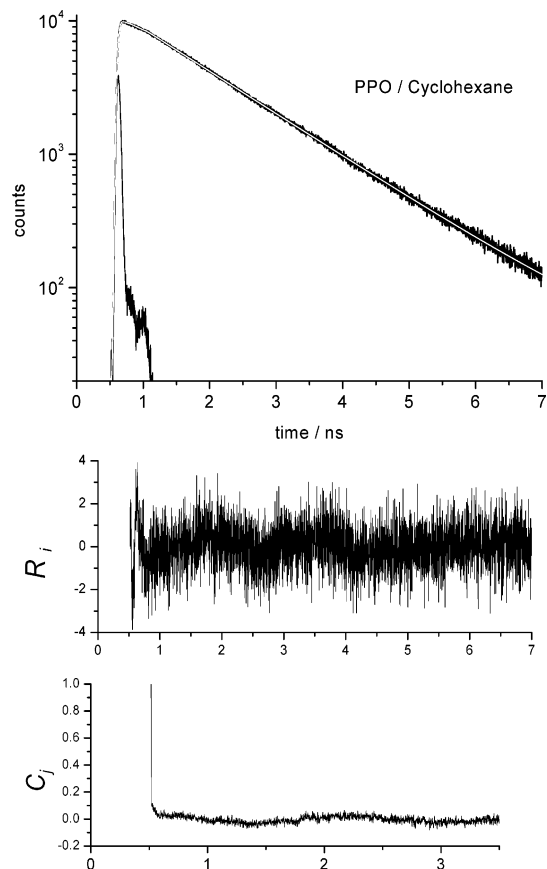
Figure 2. Data obtained by the single-photon timing technique using a mode-locked Ti:sapphire laser pumped by the second harmonic (532 nm) of a YAG laser. The output of the Ti:sapphire laser was frequency tripled to obtain the UV excitation wavelength. Experimental fluorescence decay trace of PPO in degassed cyclohexane at 20 °C (excitation wavelength λ_{ex} = 290 nm, observation wavelength λ_{em} = 380 nm, number of channels used in the fitting 3620, channel width 1.85 ps). The instrument response function $u(t)$ measured with a Ludox scattering solution in water and the best monoexponential fit to the experimental decay data are also displayed. The plot of the weighted residuals R_i versus time and the autocorrelation function C_j are given in the lower panels. Results: τ = 1.357 ± 0.002 ns, χ_v^2 = 1.072, $Z\chi_v^2$ = 3.059. The quoted error represents one standard deviation (standard uncertainty).[64]

1b). Examination of the other comparable plots reveals that the τ-values determined by BAL for NATA in water (Figure S2b, Supporting Information) and by IRV for rhodamine B in water (Figure S2c, Supporting Information) can be labeled as potential extreme values and warrant closer analysis. Numerical outlier measures that assess the degree to which an observation in a small sample is a maverick point are complimentary to simple scatter plots, which are, however, somewhat subjective. The numerical tests[65,66] for detecting outlier observations in a small sample from a normal distribution are given in the Supporting Information. These numerical tests corroborate that two outliers can be found: one in the sample of 1,9-diphenylanthracene in cyclohexane and the other for rhodamine B in water. It is clear that these outlying observations are not used in the statistical analysis.

Laboratory Bias. Possible occurrence of systematic errors in the participating laboratories can be demonstrated by using a
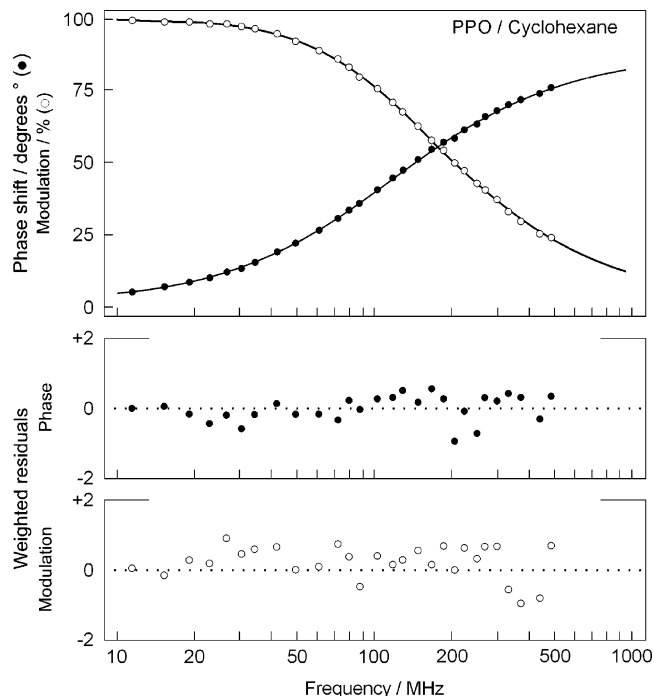

Figure 3. Experimental phase shift and modulation data of PPO in degassed cyclohexane at 20 °C (excitation wavelength λ_{ex} = 300 nm, observation wavelength λ_{em} = 360 nm) measured versus a Ludox scattering solution in water using a mode-locked laser as excitation source. The full lines show the best monoexponential fit to the experimental data. The lower panels show the deviations in phase and modulation from the single-exponential fit. Number of frequencies ω: 28, from 12 to 500 MHz. Results: τ = 1.34 ns, χ_v^2 = 1.2.

two-sample chart.[67] The plot of the estimated lifetimes for sample A against the corresponding results for sample B for each laboratory should not show any discernible trend: one should obtain a random scatter of points about a line with zero slope. However, a positive trend in the data [i.e., low (respectively high) lifetime values for sample A corresponding to low (respectively high) lifetime values for sample B] is indicative of the presence of laboratory (or method) bias. One obvious condition to plot a two-sample chart is that the same laboratory has measured the lifetimes of both samples. Additionally, the chart should have enough data points to allow a possible trend in the data to be observed. As examples we have plotted in Figure 4 two-sample charts for the estimated lifetimes of anthracene in methanol (x-ordinate) versus those of POPOP in cyclohexane (y-ordinate) (Figure 4a) and of PPO in methanol (x-ordinate) versus DPA in methanol (y-ordinate) (Figure 4b). These examples (and many others not displayed here) show that systematic errors in the lifetime determinations are absent. Indeed, linear least-squares analysis indicates that the correlation between the lifetime data sets is insignificant. With 20 different samples, the theoretical number of possible two-sample charts is very large. Pairwise comparisons of all possible lifetime data sets are not recommended because the performed tests use each data set several times (i.e., the tests are mutually dependent). Because two-sample tests do not distinguish method (TD or FD) from laboratory bias, they cannot be used to compare different methods.

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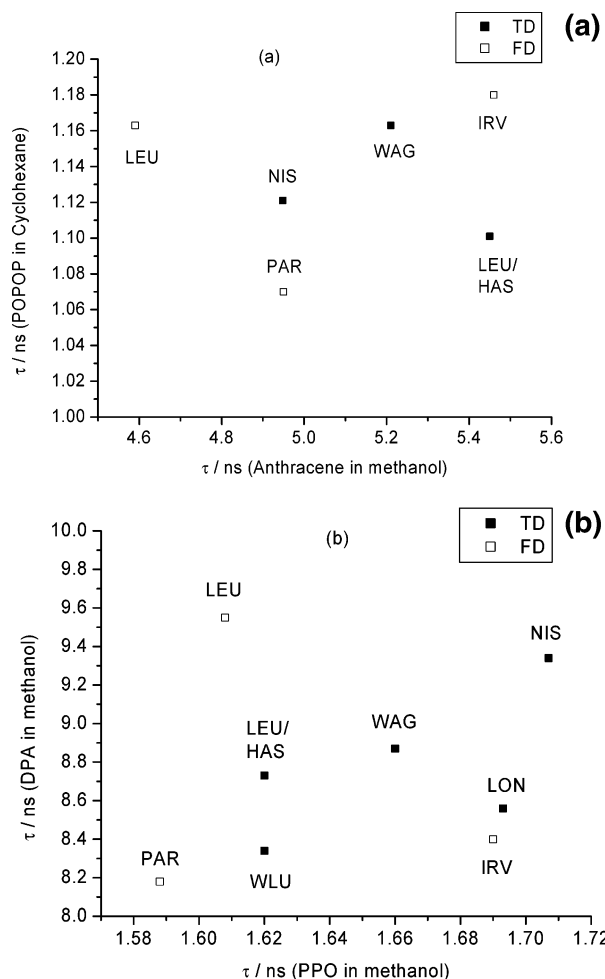
Figure 4. Two-sample method for the detection of laboratory bias. (a) Lifetime data of anthracene in methanol (*x*-ordinate) versus those of POPOP in cyclohexane (*y*-ordinate). Best linear least-squares fit to the data: $y = (1.1 \pm 0.3) + (0.009 \pm 0.063)x$ (correlation coefficient $r = 0.069$). (b) Lifetime data of PPO in methanol (*x*-ordinate) versus those of DPA in methanol (*y*-ordinate). Regression equation: $y = (5.6 \pm 7.2) + (1.9 \pm 4.3)x$ (correlation coefficient $r = 0.178$). The quoted errors represent one standard error for the intercept and the slope.

Comparison of Precision of TD and FD Methods. To know whether the two time-resolved fluorometries (i.c., TD and FD) yield the same or significantly different results, replicate analyses with each fluorometric technique were carried out by the participating laboratories. Ideally, the lifetimes obtained by both methods should be completely correlated, i.e., the correlation coefficient, *r*, should be equal to unity. However, because the correlation coefficient only gives a preliminary indication, it will not be discussed further. A *t*-test (assuming a normal distribution of errors) can be applied to investigate whether the differences between the mean lifetimes $\bar{\tau}_{TD}$ and $\bar{\tau}_{FD}$ obtained with the TD and FD methods are significant or not. The variances for the replicate analysis of each sample by the two methods can be compared using the *F*-test. These statistical tests are described in the next sections.

Replicate analysis of a sample produces the estimated variances, s_1^2 and s_2^2,[64] which are compared to test the significance of their difference. Let us assume that n_1 replicate measurements are done on a certain sample by using procedure 1 and n_2 replicate

measurements by using procedure 2. The question is whether the variances of the two populations from which the samples are drawn are different: $\sigma_1^2 \neq \sigma_2^2$. The null hypothesis, H_0, is always a hypothesis of no difference. If the null hypothesis H_0, $\sigma_1^2 = \sigma_2^2$, is true, then the estimates s_1^2 and s_2^2 do not differ very much and their ratio should be close to unity. To test this condition, one calculates the test statistic *F* as the ratio of the sample variances s_1^2 and s_2^2:

$$F = s_1^2/s_2^2 \tag{16}$$

By convention, one calculates the *F*-ratio by dividing the largest variance by the smallest. One then has to compare the calculated *F*-value with the theoretical, tabulated value $F_{(n_1-1),(n_2-1)}^{\alpha}$ at the chosen significance level α (usually 0.05 or 0.01) for $(n_1 - 1)$ and $(n_2 - 1)$ degrees of freedom. H_0 is formulated as $\sigma_1^2 = \sigma_2^2$ and H_1 as $\sigma_1^2 \neq \sigma_2^2$. H_0 is accepted (and H_1 rejected) if the calculated *F*-value is smaller than the tabulated value. If the lifetime data are normally distributed and if the variances of the two populations from which the samples are drawn are equal, a *t*-test can be performed by calculating the statistic

$$t = \frac{\bar{\tau}_1 - \bar{\tau}_2}{\sqrt{s^2[(1/n_1) + (1/n_2)]}} \tag{17}$$

which is distributed as Student's *t* with $(n_1 + n_2 - 2)$ degrees of freedom. A composite variance, s^2 in eq 17, is calculated as

$$s^2 = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2} \tag{18}$$

The calculated *t*-value is compared with the tabled, theoretical value (Table S7, Supporting Information) at the chosen significance level α for $(n_1 + n_2 - 2)$ degrees of freedom.[67] The null hypothesis H_0, $\mu_1 = \mu_2$, is accepted if the calculated *t*-value is smaller than the tabled value.

To illustrate the test for the precision of the TD and FD methods, we used the measured lifetime data of PPO in cyclohexane (Table S4, Supporting Information, and Table 2): $\bar{\tau}_1 = 1.3825$, $s_1^2 = 0.000\,603\,7$, $n_1 = 4$ (TD); $\bar{\tau}_2 = 1.346$, $s_2^2 = 0.001\,542$, $n_2 = 4$ (FD). Since the sample sizes are small, two conditions have to be fulfilled for the validity of the *t*-test. Let us assume the condition of normality of the lifetime data is met. The condition of homogeneity of the variances can be checked with the *F*-test. The calculated *F*-value (2.554) is compared to the theoretical two-tailed $F_{3,3}^{0.05}$ value at $\alpha = 0.05$ and for (3, 3) degrees of freedom, i.e., 15.44. Because this calculated value is smaller than the tabulated value, H_0, $\sigma_1^2 = \sigma_2^2$, is accepted: no difference in variance can be shown. As both conditions are fulfilled, a *t*-test can be performed with H_0, $\mu_1 = \mu_2$, and H_1, $\mu_1 \neq \mu_2$, $s^2 = 0.001\,07$, $t = 1.576$. Because the theoretical values for a two-tailed *t*-test at $\alpha = 0.05$ and for 6 degrees of freedom is 2.447 (Table S7, Supporting Information), H_0 is accepted and one can conclude that at the chosen significance level no difference between $\bar{\tau}_1$ (=$\bar{\tau}_{TD}$) and $\bar{\tau}_2$ (=$\bar{\tau}_{FD}$) can be shown. Hence, the TD and FD procedures give comparable precision. One can repeat these calculations for the

19 remaining sets of lifetime data. When only one data point is on hand via the TD (9-cyanoanthracene in cyclohexane) or FD (rhodamine B in water) methodology, no values for s^2, F, and t can be calculated. The results of the F- and t-tests for all investigated (fluorescent lifetime standard/solvent) combinations are compiled in Table 2. For each sample, the calculated F-values are smaller than the tabled two-tailed F-value at $\alpha = 0.05$, showing no difference in variance between the two time-resolved fluorescence techniques. The t-tests indicate that no difference in means can be shown. It is only for p-terphenyl in cyclohexane that the calculated t-value (2.971) is somewhat larger than the tabulated t-test value at $\alpha = 0.05$ (2.571) for 5 degrees of freedom. However, at the $\alpha = 0.01$ significance level, the tabulated Student's t-value (4.032) largely exceeds the calculated value.

An easier way of assessing the comparability of the two methods is by least-squares fitting.[67] When the mean lifetimes $\bar{\tau}_{FD}$ obtained for the samples of Table 2 with the FD procedure are plotted against $\bar{\tau}_{TD}$ obtained with the TD methodology, a straight line should be found. Theoretically, this line should have a slope, b (eq 19), of exactly unity and an intercept on the ordinate, a, of exactly zero.

$$y = a + bx \qquad (19)$$

By fitting eq 19 to the data, one obtains estimates $\hat{a}$ and $\hat{b}$ of a and b, respectively, and by a goodness-of-fit test, one can find out if eq 19 really describes the experimental observations. A caret (^) above a symbol denotes an estimate of the quantity represented by that symbol. The presence of random errors in both methods yields a scatter of points around the least-squares line and a small deviation of the estimated slope and intercept from unity and zero, respectively. Since none of the two methods yields error-free lifetime data, one should use the orthogonal regression because it takes into account errors in x and y. Indeed, the standard least-squares method's implicit assumption that one variable (y) is subject to error and the other (x) is error-free produces biased estimates of the fit parameters when that assumption is violated.[68,69] Hence, when both x and y have measurement uncertainties, the standard least-squares procedure should not be used. Instead, a general least-squares method that accounts for measurement errors on x and y should be used to perform curve fits of $\bar{\tau}_{TD}$ versus $\bar{\tau}_{FD}$ (or vice versa).[69] The best orthogonal least-squares fit (eq 19) of $\bar{\tau}_{FD}$ (y, subject to error) versus $\bar{\tau}_{TD}$ (x, subject to error) obtained for 18 samples of Table 2 gives the following estimates: $\hat{a} = -0.001 \pm 0.005$ (s_a), $\hat{b} = 1.02 \pm 0.01$ (s_b) with $r = 0.978$. Because $n_{TD} = 1$ for 9-cyanoanthracene in cyclohexane and $n_{FD} = 1$ for rhodamine B in water (Table 2), these data were excluded from the least-squares regression. Figure 5 shows the results of the best straight-line fit of $\bar{\tau}_{FD}$ (y) versus $\bar{\tau}_{TD}$ (x) when both variables contain errors. The r-value reveals that the correlation between $\bar{\tau}_{TD}$ and $\bar{\tau}_{FD}$ is very good. To investigate whether $\hat{a}$ and $\hat{b}$ differ significantly from zero and unity, respectively, one must apply t-tests. To test whether the estimate $\hat{a}$ of the intercept is significantly different from $a = 0$, one calculates $t = \hat{a}/s_a^2$ and compares this value with the tabled Student's t-distribution value t_{n-2}^α with $n - 2$ degrees of freedom at the

(68) Jefferys, W. H. *Astron. J.* **1980**, *85*, 177−181.
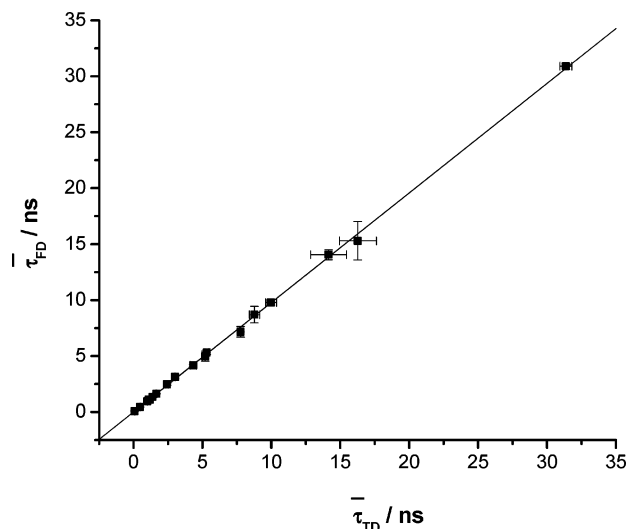(69) Lybanon, M. *Am. J. Phys.* **1984**, *52*, 22−26.

Figure 5. Linear least-squares fitting when both variables have uncertainties for assessing the comparability of the pulse (TD) and phase-modulation (FD) fluorometries. The mean $\bar{\tau}_{FD}$-values (y) obtained for the samples of Table 2 are plotted against the corresponding mean $\bar{\tau}_{TD}$-values (x). Regression equation : $y = (-0.001 \pm 0.005) + (1.02 \pm 0.01)x$ (correlation coefficient $r = 0.978$). Number of observations $n = 18$. The quoted errors represent one standard error for the intercept and the slope. The standard deviations (standard uncertainties)[64] on $\bar{\tau}_{FD}$ and $\bar{\tau}_{TD}$ are also displayed (when they are larger than the used symbols).

significance level α. The calculated t-value (0.2) is lower than the theoretical t_{16}^α-value for $\alpha = 0.05$ (2.120) or $\alpha = 0.01$ (2.921) and 16 degrees of freedom. This means that $\hat{a}$ is not significantly different from 0, implying that there is no method bias. To test whether $\hat{b}$ is significantly different from $b = 1$, one compares the calculated t-value [$t = (\hat{b} - 1)(n - 2)^{1/2}/(1 - r^2)^{1/2}$ with $n = 18$] with the tabulated Student's t-distribution value t_{n-2}^α with $n - 2$ degrees of freedom at the significance level α. Also, the t-test shows that $\hat{b}$ is not significantly different from 1 (calculated $t = 0.539$). To conclude, the values of slope, intercept, and correlation coefficient estimated by the appropriate least-squares regression of $\bar{\tau}_{FD}$ and $\bar{\tau}_{TD}$ (both variables have uncertainties) demonstrate that the TD and FD procedures have very similar precision.

DISCUSSION

For lifetime determinations of the same compound carried out by several laboratories, each with their own personnel, lifetime instrumentation, data analysis software, lifetime standards, and solvents obtained from different suppliers, etc., one expects a normal distribution of errors broader than that when an experienced analyst carries out all determinations. The dispersion around the mean lifetime $\bar{\tau}$ can be considered a measure of reproducibility. To minimize systematic errors in the fluorescence lifetime determinations, all sources of variation must be taken into account and eliminated where possible. Therefore, to create experimental conditions as similar as possible, all laboratories participating in the cooperative fluorescence lifetime standards project had to comply with the following set of preconditions. (i) Since temperature may affect the fluorescence lifetime, the temperature was set fixed at 20 °C. (ii) To avoid the quenching effect of the ubiquitous quencher oxygen, all solutions had to be deoxygenated, either by purging the solutions with N_2 or Ar or,

preferably, by degassing the solutions by repetitive freeze−pump−thaw cycles prior to the measurements. (iii) The concentration of the solute in the solution had to be low enough to avoid systematic errors that depend on the concentration of the lifetime standard (such as reabsorption, aggregate formation, etc.). Therefore, the absorbance of the solutions was kept very low (typically ∼0.05), so that the absorbed light flux was linearly proportional to the absorbance. (iv) Obviously, the impurity of both the lifetime standard and the solvent used to prepare the solutions can contribute to systematic errors. Hence, the use of standards and solvents of the highest possible purity commercially available was required. (v) To obtain reliable lifetime values, it is of utmost importance to collect high-quality experimental data, as well as to use sensitive criteria for judging the quality of the fit. High-quality data can be obtained only with state-of-the-art instrumentation that is free of systematic errors and that is expertly maintained and run. Systematic errors can be introduced into lifetime measurements by several factors [due to the fluorescent sample (purity, preparation, concentration, and geometry), the electronic and optical components of the time-resolved instrumentation, and data analysis]. Reference 13 gives an extensive list of possible error sources and their remedies in time-resolved fluorescence spectroscopy (TD and FD). All instruments used by the different groups had lasers as excitation source and possibly microchannel plate photomultipliers as photodetectors to ensure the highest temporal resolution. Actually, the use of lasers as excitation sources was chosen as an important criterion for participation in the project. Indeed, the use of a laser as excitation source was instrumental in demonstrating that quinine exhibits dual exponential fluorescence decay kinetics.[24] Before 1982, the single-exponential lifetimes of quinine solutions were "known with better accuracy than those of any other solutions described in the literature"[23] and there was "no other substance whose lifetime has been measured by so many laboratories with such good agreement as in the case of quinine".[23] Evidently, the single-exponential lifetime values measured for quinine sulfate by so many laboratories were not obtained by laser excitation. Since the signal-to-noise ratio in single-photon timing measurements is dependent on the number of counts, it is important to collect decay traces with high counts. Analogously, for the phase-modulation measurements, multiple modulation frequencies were used. (vi) Probably the surest way to minimize systematic errors in interlaboratory measurements is to select those qualified laboratories known to be particularly competent in the fields of pulse and phase-modulation fluorometry.

The small relative standard deviations[64] expressed as a percentage (Table 1) indicate that the interlaboratory uncertainty and the systematic error introduced by the use of the TD and FD methods are rather insignificant. This also is confirmed by the statistical analyses by means of two-sample charts. The unavoidable small artifacts of any instrumental setup and the difficulty of preparing identical solutions without a trace of impurity and quencher can account for the small deviations observed among the laboratories.

Our lifetime values $\bar{\tau}$ (Table 1) agree very well with the following seven reported in the literature (Table S9, Supporting Information): anthracene in cyclohexane (5.2 ± 0.1 ns, deoxygenated solutions), erythrosin B in water (0.08 ± 0.02 ns,

undegassed and deoxygenated solutions), NATA in water (3.0 ± 0.1 ns, undegassed and deoxygenated solutions), POPOP in cyclohexane (1.12 ± 0.02 ns, undegassed and deoxygenated solutions), PPO in cyclohexane (1.39 ± 0.03 ns, deoxygenated solutions), and rhodamine B in water (1.58 ± 0.08 ns, not specified whether solutions are deoxygenated) and in methanol (2.5 ± 0.3 ns, not specified whether solutions are deoxygenated). The $\bar{\tau}$ and s (u)[64] values reported here and in Table S9 are calculated for minimally three measurements. When less data were available (Table S8, Supporting Information), no averages were calculated. The shorter lifetime (4.02 ± 0.07 ns) obtained for undegassed solutions of anthracene in cyclohexane demonstrates that oxygen quenches the fluorescence by ∼20% and confirms the need of deoxygenation to obtain consistent lifetime values.

To assess the comparability of the TD and FD procedures, F-tests (eq 16), t-tests (eq 17), and linear least-squares fits (eq 19) of the mean lifetimes $\bar{\tau}_{TD}$ obtained for the samples of Table 2 with the TD procedure versus $\bar{\tau}_{FD}$ obtained with the FD procedure were performed. All these statistical tests indicate that both methods have comparable precision. Therefore, pulse (TD) and phase (FD) fluorometries are not only theoretically equivalent; they provide the same type of information [because the harmonic response is the Fourier transform of the δ-response function $f(t)$] and do this with very similar precision. From the instrumental point of view, the state-of-the-art instruments used by the research groups in this project use both lasers and microchannel plate detectors. Because the time resolution is primarily limited by the response time of the detector, this parameter is the same for both techniques. Because the methodologies are different—indeed, they are relevant to the time domain and frequency domain—it is logical that one technique will be more appropriate than the other for obtaining certain information [time-resolved spectra and anisotropy, lifetime-based decomposition of spectra, fluorescence lifetime imaging microscopy (FLIM)]. The well-defined statistics in single-photon timing is an advantage for data analysis. Time-resolved fluorescence anisotropy measurements are more straightforward in TD fluorometry. Single-photon timing has an outstanding sensitivity, for very weak fluorescent samples requiring long acquisition times this is advantageous. For single-molecule lifetime determinations, single-photon timing is the only possibility. Conversely, the short acquisition time for (ϕ, m) measurements at a single frequency is a benefit in FLIM spectroscopy, provided the fluorescence intensity is high enough to get an analog signal whose zero crossing (for ϕ measurements) and amplitude (for m measurements) can be measured with enough accuracy. For a comparison of the performance of the single-photon timing method and the frequency-domain method in two-photon FLIM, we refer to ref 70. The time of data collection depends on the complexity of the fluorescence δ-response function $f(t)$. For complicated $f(t)$, the time of data collection is approximately the same for both techniques: in TD fluorometry, a large number of timed photon events is necessary, and in FD fluorometry, a large number of frequencies has to be selected. To summarize, TD and FD fluorometries each have their own advantages and drawbacks. They appear to be complementary methods rather than competitive ones.[12]

(70) Gratton, E.; Breusegem, S.; Sutin, J.; Ruan, Q.; Barry, N. *J. Biomed. Opt.* **2003**, *8*, 381−390.

Finally, traceability is the property of the result of a measurement (i.e., fluorescence lifetime) that can be traced back to the appropriate SI unit (time, in this instance) through an unbroken chain of measurements with properly evaluated uncertainties. The international standard for time and frequency metrology is the coordinated universal time scale (UTC). Technically, the traceability of fluorescence lifetime measurements depends upon the calibration of the time (TD) or frequency (FD) axis of the participating laboratories. The quoted standard uncertainties[64] (Tables 1 and 2) imply that the contribution to uncertainty of the time (or frequency) axis calibration is marginal.

SUPPORTING INFORMATION AVAILABLE

Supporting Information Available: Absorption and fluorescence emission spectra, absorption and emission spectral maxima, molar absorption coefficients $\epsilon(\lambda_{max})$ at the maxima, fluorescence quantum yields, and literature data on all fluorescent dyes measured in the present study. Experimental conditions for the recording of the fluorescence emission spectra on the SPEX Fluorolog. Graphical and numerical criteria for assessing the quality of the fit. Numerical tests for detecting outliers. All lifetime data in liquid solution at 20 °C measured in the present study and corresponding lifetime data reported in the literature. Percentage points of T_n, percentage points of the distribution of r_{10}, and Student's t-distribution. Fluorescence decays of several dyes measured by the TD and TD methods. This material is available free of charge via the Internet at http://pubs.acs.org.