Quenched coumarin derivatives as fluorescence lifetime phantoms for NADH and FAD

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Abstract
Two-photon fluorescence lifetime imaging is a versatile laboratory technique in the field of biophotonics and its importance is also growing in the field of in vivo diagnostics for medical purposes. After years of experience in dermatology, endoscopic implementations of the technique are now posing new technical challenges. To develop, test, and compare instrumental solutions for this purpose suitable reference samples have been devised and tested. These reference samples can serve as reliable NADH- and FAD-mimicking optical phantoms for 2-photon fluorescence lifetime imaging, as they can be prepared relatively easily with reproducible and stable characteristics for this quite relevant diagnostic technique. The reference samples (mixtures of coumarin 1 and coumarin 6 in ethanol with suitable amounts of 4-hydroxy-TEMPO) have been tuned to exhibit spectral and temporal fluorescence characteristics very similar to those of NADH and FAD, the two molecules most frequently utilized to characterize cell metabolism.

KEYWORDS
coumarin 1, coumarin 6, flavin adenine dinucleotide, fluorescence lifetime imaging microscopy, multiphoton microscopy, nicotinamide adenine dinucleotide, tissue phantom

1 INTRODUCTION

Fluorescence lifetime imaging microscopy (FLIM) has developed into a versatile method and an integral constituent in biosciences and medicine. Especially imaging of NADH and FAD by means of two-photon FLIM grants new insight into cells and organisms since it allows for imaging the metabolic state of living cells, for example, in cell cultures or clinical applications, for example in dermatology [1]. To further exploit this valuable information for medical diagnostics (eg, in the form of FLIM endoscopy), new instrumentation (eg, by integration of FLIM capability into devices similar to multiphoton...
systems described in References [2, 3]) and investigation protocols need to be devised. For the development, testing or comparison of medical diagnostic systems, it is usually necessary to evaluate a system by a defined calibration protocol/procedure. This is usually done by means of artificial optical phantoms or standards with clearly defined properties, instead of tissue samples or in vivo tissue whose optical properties vary from sample to sample, site to site, or patient to patient, thus rendering reproducible measurements impossible. Artificial optical phantoms with reproducible and tunable properties are necessary in order to ensure reproducibility, stability and comparability [4] of results obtained under different conditions or with different instrumentation (for example different research microscopes, systems comprising different detector types or components from different manufacturers, different software, or different classes of optical devices, for example, standard microscope objectives vs endoscopes.

Indeed, NADH and FAD for themselves prove to be unsuitable as standards for comparing different measurement systems because of their limited shelf life in solution and the dependence of their fluorescence characteristics on external conditions, such as pH value [5] and oxidative state (NADH fluorescence intensity decreases, FAD fluorescence intensity increases with oxidation, for example, within hours in solutions exposed to air). Furthermore, with regard to their protein-bound and unbound forms, pH value and concentration, the number of exponential functions and fluorescence lifetime values regarded as required to properly fit their fluorescence decay curves varies in literature in contrast to other substances proposed as standards, like Rose Bengal, 2,5-Diphenyloxazole (PPO), 1,4-Bis(5-phenyl-2-oxazolyl)benzene (POPOP), or anthracene. Such variations and uncertainties are not desired for a standard or reference sample that should deliver stable measurement results under different conditions.

2 | MATERIAL AND METHODS

2.1 | Experimental setup for two-photon FLIM

The employed instrumental setup [6], equipped with different optical filters, is shown in Figure 1. It consists of a multiphoton laser scanning microscope (LSM 710 NLO, Carl Zeiss Microscopy GmbH, Jena, Germany) combined with a time-correlated single-photon counting (TCSPC) system based on two HPM-100-40 detectors (Becker & Hickl GmbH, Berlin, Germany), attached to a non-descanned (NDD) sideport of the LSM 710, and a Simple-TAU 152 TCSPC system, controlled by a separate computer running SPCM Ver. 9.61 32 Bit (Becker & Hickl GmbH, Berlin, Germany). For two-photon excitation, a pulsed femtosecond titanium-sapphire laser (Ti:Sa) (MaiTai AX HPDS, Spectra Physics, Darmstadt, Germany) was used, providing <100 fs pulse width and a wavelength tuning range of 690 to 1040 nm. The laser power entering the LSM 710 is adjusted by an acousto-optic modulator (AOM), controlled by the microscope software ZEN (Carl Zeiss Microscopy GmbH, Jena, Germany). It is expressed as a percentage (0%-100%), defined by the ratio of the light power leaving and entering the AOM, respectively. The absolute light power in the focal plane was measured using a powermeter (S130C with PM100D, Thorlabs Inc., Newton).

The generated fluorescence signal is cleaned up at the NDD port by a 355 to 690 nm bandpass filter, further separated by a 490 nm beam splitter, and directed to the SPC modules. For FAD and NADH detection, two SPC modules are used in combination with a 562/40 nm bandpass filter and a 436/20 nm bandpass filter (both AHF Analysentechnik, Tübingen, Germany) as described in Rück et al. [7], respectively. The instrumental setup used for the development of an NADH/FAD phantom was also used for experiments on cell cultures and chosen for compatibility and comparability.

Fluorophore solutions were filled into microscopy-suited channels (μ-Slide VI 0.4, Cat.No. 80606, ibidi GmbH, Gräfelfing, Germany) with a capacity of 40 μL per channel, closed by plugs (Luer Plug, Cat.No: 10822, ibidi, Gräfelfing, Germany) to minimize evaporation during measurements. All experiments were carried out at room temperature (22°C). Fluorescence lifetime measurements were performed at (200 ± 5) μm depth in the vertical center of the channels using an x20 objective.
different fluorophore solutions with literature values. The fluorophores, excitation wavelengths, detection filters, concentrations, solvents, and the number of measurements per fluorophore are listed in Table 1.

The fluorophore solutions were filled into microscopy-suited channels as described above. The laser excitation power was chosen individually for each fluorophore, according to the specific fluorescence intensity, in order to protect the TCSPC detectors from overload/destruction. Data analysis was done using SPCImage individually for PPO and Rose Bengal, for all other fluorophores one dataset was fitted using SPCImage and the same settings (bin, threshold, instrumental response function [IRF]) reused by batch-processing for all other datasets of the same fluorophore.

2.2 Validation of FLIM-system

The FLIM system’s correct function was validated by comparing the measured fluorescence lifetimes of six

| Fluorophore | Distributor | Article no. | mol L⁻¹ | Solvent | λ_ex (nm) | Filter | n |
|-------------|-------------|-------------|---------|---------|-----------|--------|---|
| Coumarin 6  | Sigma Aldrich | 546 283 | 2 · 10⁻⁴ | Ethanol | 780 | 562/40 | 3 |
| Coumarin 6  | Sigma Aldrich | 546 283 | 2 · 10⁻⁴ | Ethanol | 880 | 562/40 | 3 |
| PPO         | Sigma Aldrich | D210404 | 10⁻³  | Ethanol | 780 | 436/20 | 2 |
| POPOP       | Sigma Aldrich | P3754 | Saturated | Ethanol | 780 | 436/20 | 7 |
| Rose Bengal | Sigma Aldrich | 330 000 | 10⁻³  | Methanol | 780 | 562/40 | 5 |
| Rose Bengal | Sigma Aldrich | 330 000 | 0.5 · 10⁻³ | Water | 780 | 562/40 | 2 |
| Ethanol     | Merck       | Uvasol | 1.0098 |         |           |        |   |
| Methanol    | Merck       | Uvasol | 1.06002 |         |           |        |   |
| Water       | Merck       | LiChrosolv | 1.15333 |         |           |        |   |

Note: mol L⁻¹: concentration; λ_ex: excitation wavelengths; Filter: detection filters (center wavelength/full width in nm); n: number of fluorescence lifetime measurements per fluorophore.
ethanol (spectroscopy grade) in comparison to freshly prepared solutions of NADH and FAD in TRIS buffer. For this purpose, single-photon absorption spectra were measured with a UV/VIS spectrometer (Lambda 40 P, Perkin Elmer, Rodgau, Germany), single-photon (1P) excitation and emission spectra were measured with a fluorescence spectrometer (Fluoromax II, Horiba, Tokyo, Japan), and two-photon (2P) emission spectra were measured with a multiphoton microscope (Zeiss LSM710 NLO, Carl Zeiss Microscopy GmbH, Jena, Germany).

The measured spectra are shown in Figure 2 for NADH and coumarin 1 and in Figure 3 for FAD and coumarin 6. The wavelengths used for 1P and 2P excitation and the detection filter regimes are indicated in the graphs.

2.4 Determination of the dependence of coumarin fluorescence lifetimes on fluorophore concentration

For coumarin 6, the fluorescence lifetime is reported to be independent of fluorophore concentration in the range of 10⁻² to 10⁻⁶ mol L⁻¹ [12]. It was checked whether this behavior can also be confirmed for coumarin 1, for 10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵ mol L⁻¹ solutions in ethanol at excitation wavelength 780 nm. In the case of coumarin 6, only selected measurements were performed to confirm the information already available from literature [12].

2.5 Determination of the dependence of coumarin fluorescence lifetimes on quencher concentration

The dependencies of the fluorescence lifetimes of coumarin 1 and coumarin 6 in ethanol on the concentration of the quencher 4-hydroxy-TEMPO were determined by two-photon fluorescence lifetime imaging. Suitable fluorophore concentrations, excitation wavelengths and laser power settings were chosen in each case. The choices made are discussed in the respective sections. From coumarin 1, a 2 × 10⁻² mol L⁻¹ solution in ethanol was prepared and further diluted to 2 × 10⁻⁴ mol L⁻¹. A 4-hydroxy-TEMPO solution (CL) or one of the eight prepared 4-hydroxy-TEMPO solutions (C1.1-C1.8) was added to 2 × 10⁻⁴ mol L⁻¹ coumarin 1 solution with either pure ethanol or 5 × 10⁻³ mol L⁻¹ TRIS buffer. Nine solutions named C1.0 to C1.8 with constant coumarin 1 molality of 10⁻⁴ mol L⁻¹ and increasing 4-hydroxy-TEMPO molality of 10⁻⁴ to 10⁻² mol L⁻¹ were obtained by mixing equal volumes of coumarin 1 solution with ETADPO solutions. The 4-hydroxy-TEMPO was prepared and diluted into a 0.83 mol L⁻¹ solution of NADH and FAD. The 4-hydroxy-TEMPO solution was prepared to 10⁻⁴ mol L⁻¹ and increasing concentrations of 4-hydroxy-TEMPO were obtained by mixing equal volumes of two different solutions of different molarities. Nine solutions named C1.0 to C1.8 with constant 4-hydroxy-TEMPO molality of 10⁻⁴ mol L⁻¹ and increasing coumarin 1 molality of 10⁻⁴ to 10⁻² mol L⁻¹ were obtained by mixing equal volumes of coumarin 1 solution with 4-hydroxy-TEMPO solutions. Nine solutions named C1.0 to C1.8 with constant 4-hydroxy-TEMPO molality of 10⁻⁴ mol L⁻¹ and increasing coumarin 1 molality of 10⁻⁴ to 10⁻² mol L⁻¹ were obtained by mixing equal volumes of coumarin 1 solution with 4-hydroxy-TEMPO solutions. Three solutions named C1.0 to C1.8 with constant coumarin 1 molality of 10⁻⁴ mol L⁻¹ and increasing 4-hydroxy-TEMPO molality of 10⁻⁴ to 10⁻² mol L⁻¹ were obtained by mixing equal volumes of coumarin 1 solution with 4-hydroxy-TEMPO solutions. The fluorescence lifetimes of the solutions C1.0 to C1.8 are listed in Table 3.

| Fluorophore | Distributor | Article no. | mol L⁻¹ | Solvent       | λex (nm) | Filter          | n     |
|-------------|-------------|-------------|---------|---------------|----------|----------------|-------|
| Coumarin 1  | Sigma Aldrich | D87759      | *       | Ethanol       | 780, *   | 436/20         | ♦     |
| Coumarin 6  | Sigma Aldrich | 546 283     | *       | Ethanol       | 780, 880, * | 436/20, 562/40 | ♦     |
| NADH        | Sigma Aldrich | N8129       | 5 × 10⁻³ | TRIS buffer   | 780, *   | 436/20         | 16    |
| FAD         | Enzo Life Sciences, Inc. | ALX-480-084-M050 | 5 × 10⁻³ | TRIS buffer   | 780, 880 | 562/40         | 3     |
| FAD         | Sigma Aldrich | F6625       | *       | TRIS buffer   | *        | *              | -     |

Note: mol L⁻¹: concentration; λex: excitation wavelengths; Filter: detection filters (center wavelength/full width [in nm]); n: number of fluorescence lifetime measurements per fluorophore; ♦: different values, depending on experiment (see main text); -: not applicable; ■: used in single-photon (absorption and single-photon fluorescence) experiments; *: used in multiphoton (fluorescence intensity and/or lifetime) experiments; ♦: used in multiphoton (fluorescence intensity and/or lifetime) and single-photon (absorption and single-photon fluorescence) experiments.
FIGURE 2  Absorption and fluorescence data for NADH and coumarin 1, for explanation of the curves refer to legend; 1P: single-photon data, 2P: two-photon data; A, abs: absorption spectrum; fluor. ex.: fluorescence excitation spectrum; $\lambda_{ex}$: half two-photon excitation wavelength; fluorescence excitation spectra scaled to fit absorption spectra; B, fluor. em.: fluorescence emission spectrum; black dashed lines: 436/10 nm BP, black dash-dot lines: 562/40 nm BP; data normalized to maximum

FIGURE 3  Absorption and fluorescence data for FAD and coumarin 6, for explanation of the curves refer to legend; 1P: single-photon data, 2P: two-photon data; A, abs: absorption spectrum; fluor. ex.: fluorescence excitation spectrum; $\lambda_{ex}$: half two-photon excitation wavelength; fluorescence excitation spectra scaled to fit absorption spectra; B, fluor. em.: fluorescence emission spectrum; black dashed lines: 436/20 nm BP, black dash-dot lines: 562/40 nm BP; data normalized to maximum
All nine solutions C1.0 to C1.8 were prepared independently three times (further referred to as batch 1, 2 and 3) from scratch on separate days. The fluorescence lifetime of each solution was measured one to three times, in each case with two different relative laser power settings (2% and 3%, referring to 1.1 mW and 2 mW, as measured in the focal plane) at \( \lambda_{\text{ex}} = 780 \text{ nm} \).

For coumarin 6, fluorescence lifetime measurements with two-photon excitation at 780 and 880 nm were performed on solutions with coumarin 6 and 4-hydroxy-TEMPO concentrations as shown in Table 4. The relative laser power used for both excitation wavelengths was between 1% and 2% (referring to 0.1 to 0.5 mW in the focal plane for \( \lambda_{\text{ex}} = 880 \text{ nm} \) and 0.3 to 1.1 mW for \( \lambda_{\text{ex}} = 780 \text{ nm} \)). Every measurement was performed once, except in the absence of quencher, where three measurements at 780 nm and two measurements at 880 nm were performed.

Fluorescence lifetime data were recorded for 30 s in each case. The resulting data were analyzed using SPCImage 8.0 NG for coumarin 1 and SPCImage 8.1 NG for coumarin 6 with the fit method weighted least square (WLS). From each of the three coumarin 1 batches one of the obtained fluorescence lifetime datasets was analyzed using SPCImage by adjusting bin factor and threshold. The IRF was automatically calculated by SPCImage at the brightest pixel of the time-integrated image. All other fluorescence lifetime datasets of the same coumarin 1 batch were then analyzed using SPCImage’s batch processing function, using the same bin factor, threshold and IRF for all datasets of that batch. Datasets of coumarin 6 where analyzed in a similar way, yet with the IRF calculated at the brightest pixel of each individual time-integrated image. A monoexponential decay model turned out to be sufficient in each case.

For the analysis of coumarin 1 fluorescence lifetime images using SPCImage, a binning factor of 1 was used and the decay model option was set to incomplete, to account for residual fluorescence from the previous excitation pulse [13]. The mean value \( \mu \) and the two values \( \mu \pm \sigma \) derived from the lifetime distribution histogram was exported for every dataset. Six fluorescence lifetime results were obtained for each of the solutions C1.0 to C1.8 (three repetitions for each of the two relative laser power settings 2% and 3%) and were imported into Matlab (The MathWorks Inc., Natick, Massachusetts, United States; Version 2020a) for statistical analysis, further fitting operations, and visualization. For each of the nine samples C1.0 to C1.8 with different quencher concentrations \( Q \), the median \( \tau_{B1-3} \) and the SD \( \sigma_{B1-3} \) derived from the three batches were calculated from the six fluorescence lifetime values. The fluorescence lifetime \( \tau_0 \) of unquenched coumarin 1 was defined as the mean value of the six fluorescence lifetime values obtained on solution C1.0 in Table 3. Basic solutions as well as the solutions C1.0 to C1.8 were prepared in tubes (Safe-Lock Tubes 2 mL, Eppendorf AG, Hamburg, Germany) and after the initial experiment the remaining volume was kept refrigerated between 2 and 4 °C for later analysis, after being sealed with Parafilm M (Bemis Company, Inc, Oshkosh, United States).

The fluorescence lifetime \( \tau_0 \) for unquenched coumarin 6 was defined as the mean fluorescence lifetime value of the five fluorescence lifetime values obtained in the absence of quencher and compared to the coumarin 6 fluorescence lifetime values obtained from the systems validation described above. The procedure for the analysis of coumarin 6 fluorescence lifetime images of the samples C6.0 to C6.7 was basically the same as described for coumarin 1, except for the usage of a bin factor of 4 in images with high quencher concentrations. Results were again averaged over different relative laser power settings (in this case ranging between 1% and 2% for both two-photon excitation wavelengths).

To check if the non-transparent quencher (stock solution has dark amber color for the human visual system) has a detectable influence on the fluorescence spectrum, the following procedure was performed for each of the coumarin 1 samples C1.0 to C1.8 and coumarin 6 samples C6.0, C6.3, and C6.7. A fluorescence spectrum was recorded in the same focal plane as the acquired FLIM data, using the LSM 710’s spectral imaging unit, an excitation wavelength of 780 nm for coumarin 1 and additionally 880 nm for coumarin 6. It was verified that the spectrum did not contain relevant contributions attributable to the quencher.

Additionally it was checked if the quenched and non-quenched coumarin 1 and coumarin 6 solutions show a monoexponential fluorescence decay behavior. Firstly, by switching to a double-exponential fitting model with a second decay component, observing the residuals, the

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Identifier} & \text{C1.0} & \text{C1.1} & \text{C1.2} & \text{C1.3} \\
\text{mol L}^{-1} 4\text{-hydroxy-TEMPO} & 0 & 2.6 \times 10^{-5} & 5.2 \times 10^{-4} & 1.0 \times 10^{-2} \\
\hline
\text{Identifier} & \text{C1.4} & \text{C1.5} & \text{C1.6} & \text{C1.7} & \text{C1.8} \\
\text{mol L}^{-1} 4\text{-hydroxy-TEMPO} & 2.6 \times 10^{-2} & 0.1 & 0.21 & 0.415 \\
\hline
\end{array}
\]

**Table 3** Coumarin 1/4-hydroxy-TEMPO combinations

*Note: Coumarin 1 concentration: \( 10^{-4} \text{ mol L}^{-1} \) for all combinations.*
goodness of fit $\chi^2$, and the relative contribution of the second component to the fit model [13]. Second, by using the phasor plot of SPCImage and confirming that the phasors are concentrated in one narrow spot on or very close to the semicircle of the phasor plot [13, 14].

### 2.6 | Fitting functions for coumarin 1 and coumarin 6 fluorescence quenching

The dependencies of the obtained fluorescence lifetime values $\tau_{B1-3}$ on quencher concentration were analyzed by fitting different model functions to the observed datapoints, using Matlab’s Curve Fitting Toolbox (version 3.5.10, The Mathworks Inc).

The Stern-Volmer equation for dynamic quenching [15–17],

$$F_0/F = \frac{\tau_0}{\tau} = 1 + K_{SV}[Q]$$

(1)

Further referred to as SV1, describes the reduction of the fluorescence intensity $F$ and the fluorescence lifetime $\tau$ in the presence of a quencher with concentration $Q$ [mol L$^{-1}$], where $\tau_0$ and $F_0$ are the corresponding values without quencher and $K_{SV}$ the Stern-Volmer quenching constant. This equation can alternatively be written as a hyperbolic function,

$$\tau = \frac{\tau_0}{1 + K_{SV,H1}[Q]}$$

(2)

Further referred to as H1, as far as the fluorescence lifetime $\tau$ is concerned. The intensity term $F_0/F$ was disregarded here, since fluorescence intensities were not examined or evaluated quantitatively.

By adding a quadratic term in the denominator on the right-hand side of Equation (2), a refined version of the hyperbolic function H1 can be constructed,

$$\tau = \frac{\tau_0}{1 + K_{SV,H1}[Q] + K_{SV2,H1}[Q]^2}$$

(3)

Further referred to as H2.

In case of combined static and dynamic quenching, the quenching of the fluorescence intensity can be described by the following extended version, Equation (4), of the aforementioned Stern-Volmer equation SV1 for dynamic quenching [15]:

$$\frac{F_0}{F} = (1 + K_D[Q])(1 + K_S[Q])$$

(4)

An analogously formulated phenomenological equation for the fluorescence lifetime,

$$\frac{\tau_0}{\tau} = (1 + K_D[Q])(1 + K_S[Q])$$

(5)

Further referred to as SV2, has also been tested to fit the obtained dependence of fluorescence lifetimes on the quencher concentration.

### 2.7 | Adjustment of phantom fluorescence lifetime to in-vivo NADH and FAD

From the model fitting of the observed $\tau_{B1-3}(Q)$ datasets for coumarin 1 based on Equation 1, the 4-hydroxy-TEMPO concentrations $Q_{0.4}$ and $Q_{2.5}$ were determined that correspond to the fluorescence lifetimes of free and bound NADH, $\tau_1 \approx 0.4$ ns [18, 19] and $\tau_2 \approx 2.5$ [16], respectively. Subsequently, two solutions of $10^{-4}$ mol L$^{-1}$ coumarin 1 and the two concentrations $Q_{0.4}$ and $Q_{2.5}$ of 4-hydroxy-TEMPO were prepared as described above, and the fluorescence lifetimes were measured at 2% and 3% relative laser power at 780 nm excitation wavelength three times each, using the same conditions as for the determination of $\tau_{B1-3}$. The two solutions were prepared three times from scratch, and measurements were repeated, leading to 18 fluorescence lifetime values per solution. Mean, median, and SD were determined using Matlab, and the values were compared to the two target fluorescence lifetime values.

To mimic the fluorescence lifetimes of bound and free FAD, $\tau_1 \approx 0.1$ ns (106 ps [20], 130 ps [21]) and $\tau_2 \approx 2$ ns (see Table 6), target lifetimes of $\tau_1 = 0.1$ ns and $\tau_2 = 2$ ns were chosen for the coumarin 6 solution. From the model

| Identifier | C6.0 | C6.1 | C6.2 | C6.3 |
|------------|------|------|------|------|
| mol L$^{-1}$ 4-hydroxy-TEMPO | 0 | 1.14 $\times$ 10$^{-2}$ | 2.08 $\times$ 10$^{-2}$ | 8.30 $\times$ 10$^{-2}$ |
| mol L$^{-1}$ Coumarin 6 | $10^{-4}$ and 7.5 $\times$ 10$^{-5}$ | 7.5 $\times$ 10$^{-5}$ | 7.5 $\times$ 10$^{-5}$ | 10$^{-4}$ |

| Identifier | C6.4 | C6.5 | C6.6 | C6.7 |
|------------|------|------|------|------|
| mol L$^{-1}$ 4-hydroxy-TEMPO | 0.114 | 0.208 | 0.396 | 0.415 |
| mol L$^{-1}$ Coumarin 6 | 7.5 $\times$ 10$^{-5}$ | 7.5 $\times$ 10$^{-5}$ | 7.5 $\times$ 10$^{-5}$ | 10$^{-4}$ |
fitting of the observed $\tau(Q)$ datasets for coumarin 6 based on Equation (3), the 4-hydroxy-TEMPO concentration $Q_{2,0}$ was determined that corresponds to the fluorescence lifetime of free FAD, $\tau_2 \approx 2.0$ ns. For the fluorescence lifetime $\tau_1 = 0.1$ ns of bound FAD, the required quencher concentration was not within the examined range. Nevertheless, by extrapolation based on fit function H2, the hypothetically required quencher concentration $Q_{0,1}$ could be calculated to mimic $\tau_1 = 0.1$ ns of bound FAD. Since it is unknown if a fluorescence lifetime of $\approx 0.1$ ns can be reached by quenching coumarin 6, an alternative substance is proposed for this purpose.

### 2.8 Test of coumarin 1 long-time stability

Since long-time stability is highly desirable for optical phantoms, the fluorescence lifetime $\tau_{92,\text{Rep}}$ (repetition measurement) of the nine preserved coumarin 1 solutions C1.0 to C1.8 of batch 2 were measured again after 41 days. Additionally, the preserved basic solutions for coumarin 1 batch 2 ($2 \times 10^{-4}$ mol L$^{-1}$ coumarin 1 solution and the 4-hydroxy-TEMPO solution with molarity 0.83 mol L$^{-1}$ plus seven diluted versions thereof) were still available. By mixing them again at equal volumes, the initial preparation was recreated, yielding fluorescence lifetimes $\tau_{92,\text{New}}$ (measurement after new preparation). All solutions were measured once at 780 nm and for 2% and 3% relative laser power. Fitting with SPCImage was done using the same values for threshold and binning factor as for the initial measurements.

### 3 RESULTS

#### 3.1 Validation of FLIM system

In Table 5, results of fluorescence lifetime measurements on fluorescence lifetime standards proposed in literature are compared with the corresponding literature values. The multiphoton FLIM system used for the present work seems to provide fluorescence lifetimes in good agreement with the ones expected from literature. All fluorophores listed in Table 5 showed monoexponential decay behavior, as expected from literature.

#### 3.2 NADH and FAD fluorescence lifetimes

The measured fluorescence lifetimes of unbound NADH and FAD solutions in 1 mol L$^{-1}$ TRIS pH 7.5, prepared as specified in Table 2, are shown in Table 6. The obtained fluorescence lifetimes for NADH are in reasonable agreement with literature. For FAD the obtained fluorescence lifetime corresponds to one of the published lifetimes of $\approx 2000$ ps. Reported fluorescence lifetimes of FAD, which could exist protein-bound and unbound, vary in the range 7 to $\approx 4000$ ps [5, 28, 29], depending on stacking and conformation. A fluorescence lifetime of $\approx 2000$ ps for FAD is usually associated with free FAD [5, 15, 21, 30], therefore the same molecule state was assumed here.

Fitting was done monoexponentially also for NADH since no indication of bi- or tri-exponential decay behavior was discernable in the data, and the obtained fluorescence lifetime $\approx 400$ ps was interpreted as indication that the fluorophore was present in its free (unbound) form [19, 30, 31]. FAD was fitted monoexponentially since the presence of a second or third component could not be verified with absolute certainty.

#### 3.3 Dependency of coumarin 1 and coumarin 6 fluorescence lifetimes on fluorophore concentration

Independence of the fluorescence lifetime on coumarin 1 concentration in ethanol could be confirmed for concentrations $10^{-3}$, $10^{-4}$, $10^{-5}$ mol L$^{-1}$, and a mean value of $(2913 \pm 52)$ ps was obtained from a total of 11 measurements. For a coumarin 1 concentration of $10^{-2}$ mol L$^{-1}$, which in contrast to coumarin 6 at this concentration [12] dissolved completely, the fluorescence lifetime was $\approx 2700$ ps. The independence of the fluorescence lifetime of coumarin 6 on concentration reported in literature [12] could be confirmed for all tested fluorophore concentrations.

#### 3.4 Dependency of coumarin 1 and coumarin 6 fluorescence lifetimes on quencher concentration

By means of the quencher 4-hydroxy-TEMPO, the fluorescence lifetime of coumarin 1 could be varied in a range between $\tau_0 = (2927.8 \pm 16.9)$ ps in the absence of quencher and $\tau = (196.8 \pm 12.4)$ ps at a quencher concentration of 0.415 mol L$^{-1}$. Likewise, the fluorescence lifetime of coumarin 6 could be varied between $\tau_0 = (2478.8 \pm 36.1)$ ps and $\tau = 200$ ps. In all cases, the monoexponential decay behavior was maintained. The determined value $\tau_0$ for coumarin 6 in these quenching experiments lies within the SD of the coumarin 6 measurements performed for the validation of the FLIM system (Table 5).
In Figure 4 the fit functions H1 and H2 defined in Equations (2) and (3), respectively, are shown together with the measured fluorescence lifetimes of coumarin 1 ($\tau_{B1-3}$, the average over sample batches 1-3) and coumarin 6. Fitting with fit function H1 yields $K_{SV,H1} = 22.73 \text{ (20.89,24.57) L mol}^{-1}$ with $R^2 = 0.998$ for coumarin 1 and $K_{SV, H1} = 20.00 \text{ (18.62,21.37) L mol}^{-1}$ with $R^2 = 0.998$ for coumarin 6. Here the 95% confidence intervals are added in brackets. Despite the high $R^2$ value in both cases, the fits in Figure 4 show good agreement with the measured data only for lower quencher concentrations up to $\approx 0.1 \text{ mol L}^{-1}$, while increasing deviations of the H1 fit curves from the datapoints are observed for higher quencher concentrations, both in the case of coumarin 1 and coumarin 6. Since it was strongly desired that the fit model is valid also for short fluorescence lifetimes of 0.1 ns to 0.4 ns, which turned out to require higher quencher concentrations $>0.1 \text{ mol L}^{-1}$, this problem cannot be neglected. It was therefore addressed by modification of the fitting model, leading to the fit function H2 defined in Eq. (3). The respective curve fits yielded the model parameters $K_{SV, H2} = 20.36 \text{ L mol}^{-1}$ and $K_{SV^2, H2} = 32.43 \text{ L}^2 \text{ mol}^{-2}$ and $R^2 = 0.999$. In contrast to H1, the H2 fit curves are in good agreement with the coumarin 1 and coumarin 6 fluorescence lifetime datapoints within the entire examined range of quencher concentrations, including quencher concentrations $>0.1 \text{ mol L}^{-1}$ (see Figure 4). Figure 5 shows the Stern-Volmer plots, $\tau_0 \tau^{-1}$ vs quencher concentration $Q$, for the measured fluorescence lifetimes of coumarin 1 and coumarin 6, in each case together with the fit functions SV1 and SV2 defined in Equations (1) and (5), respectively. The fit of the linear Stern-Volmer relationship in Equation (1) for solely dynamic quenching yields $K_{SV} = 31.65 \text{ (29.01,34.29) L mol}^{-1}$ and $K_{SV2} = 32.43 \text{ L}^2 \text{ mol}^{-2}$ and $R^2 = 0.999$. In contrast to H1, the H2 fit curves are in good agreement with the coumarin 1 and coumarin 6 fluorescence lifetime datapoints within the entire examined range of quencher concentrations, including quencher concentrations $>0.1 \text{ mol L}^{-1}$ (see Figure 4).

### Table 5

| Fluorophore         | $n$ | $\tau$ (ps) | $\tau - \sigma$ (ps) | $\tau + \sigma$ (ps) | Lit. $\tau$ (ps) |
|---------------------|-----|-------------|-----------------------|-----------------------|------------------|
| PPO                 | 2   | 1617.51     | 1595.43               | 1639.9                | 1600 [15]        |
|                     |     | 1612.99     | 1582.1                | 1643.5                |                  |
| Mean $\tau$ (ps)   |     | 2488.93     | 41.92                 | 2390 [12]             |                  |
| STD $\tau$ (ps)    |     |             |                       |                       |                  |
| Coumarin 6          | 6   | 2488.93     | 41.92                 | 2390 [12]             |                  |
| POPOP               | 7   | 1273.49     | 16.75                 | 1430 [22], 1400 [23]  |                  |
| Rose Bengal (Methanol) | 5  | 555.22      | 4.83                  | 519 [15], 516 [24], 540 [25] | |
| Rose Bengal (Water) | 4   | 87.1        | 6.6                   | 140 [26], 120 [25], 78 [27] | |

**Note:** $n$: number of fluorescence lifetime measurements per fluorophore.

### Table 6

| Fluorophore         | $n$ | Mean $\tau$ (ps) | STD $\tau$ (ps) | Lit. $\tau$ (ps) |
|---------------------|-----|------------------|-----------------|------------------|
| NADH                | 16  | 416.9            | 26.3            | $\approx$400 [18, 19], 470 [31], 500 ps [22] |
| FAD                 | 3   | 1945.4           | 34.7            | $\approx$2000 [20], $r_3 = 2090 \text{ (solution)}$ [5], 2460 [22], 2300 ± 700 [21], $r_2 = 2820 \text{ (solution)}$ [28], 2470 [32] |
the measured fluorescence lifetime datasets (see SV1 curves in Figure 5). The fits to the quadratic function SV2 defined in Equation (5) yield $K_S = 19.18 (18.6,19.76)$ L mol$^{-1}$, $K_D = 1.60 (1.479,1.706)$ L mol$^{-1}$ and $R^2 = 1.0$ for coumarin 1, and $K_S = 16.61 (13.74,19.48)$ L mol$^{-1}$, $K_D = 1.189 (0.6257,1.752)$ L mol$^{-1}$ and $R^2 = 0.998$ for coumarin 6. In both cases, the measured datapoints are adequately described by the fit function SV2 in the entire range of examined quencher concentrations, as in the case of Figure 4 and the fit function H2 defined in Equation (3).

Two-photon fluorescence emission spectra of coumarin 1 and coumarin 6 with concentrations listed in Tables 3 and 4, respectively, showed a decrease in fluorescence intensity with increasing quencher concentration as to be expected from the Stern-Volmer relationship in Equation (1). For both substances no shift in fluorescence wavelength with quencher concentration and, in the case of coumarin 6, excitation wavelength could be observed.

3.5 NADH and FAD fluorescence lifetime synthesis

For the synthesis of phantom solutions with fluorescence lifetimes corresponding to those of free and bound NADH, $\tau_1 \approx 0.4$ ns and $\tau_2 \approx 2.5$ ns, respectively, the required 4-hydroxy-TEMPO concentrations were determined as $Q_{\tau_1} = 0.234$ mol L$^{-1}$ and $Q_{\tau_2} = 8.55 \times 10^{-3}$ mol L$^{-1}$, based on the fit function H2 defined in Equation (3).

From the 18 measurements per target fluorescence lifetime, the mean, median and SD were calculated using Matlab as shown in Table 7. In the case of both NADH variants, the obtained fluorescence lifetime is shorter than the target value, but still within the range of the SD.

For the synthesis of phantom solutions with fluorescence lifetimes corresponding to those of bound and free FAD, $\tau_1 \approx 0.1$ ns and $\tau_2 \approx 2.0$ ns, respectively, the required 4-hydroxy-TEMPO concentrations were determined as $Q_{\tau_2} = 1.3 \times 10^{-3}$ mol L$^{-1}$ and, outside of the examined Q range, $Q_{\tau_1} = 0.713$ mol L$^{-1}$, again based on the fit function H2.

3.6 Test of long-time stability of coumarin 1 solutions

The fluorescence lifetime values $\tau_{B1-3}$ obtained from the initial measurements on sample batches 1 to 3 where compared with the results of measurements performed 41 days later. The values $\tau_{B2,Rep}$ were obtained in repeated measurements on the archived samples of batch 2 and the values $\tau_{B2,New}$ were obtained on samples newly mixed from the archived basic solutions of batch 2.

The fluorescence lifetimes $\tau_{B2,Rep}$ and $\tau_{B2,New}$ obtained after 41 days are consistent with those obtained in the initial measurements. The maximum deviation from the initial results $\tau_{B1-3}$ was found to be $\Delta \tau = 36.8$ ps. This indicates that a solution prepared and stored in
the described way is at least stable within the time frame of 1 month. This would be consistent with the reported finding that coumarin 6 in ethanol is stable as a fluorescence lifetime standard for months [14].

4 | DISCUSSION

4.1 | General data processing

For the analysis of FLIM data, one can and has to choose between a vast number of adjustable fit options beforehand. The SPCImage software provides three different fitting methods (one of which may alternatively be implemented on GPU or CPU), 11 different binning factors (0 and 10), usually three different types of fitting function (single, double, triple exponential), and two settings for the decay model (complete and incomplete), which already makes up for at least $3 \times 11 \times 3 \times 2 = 198$ different configurations of the fitting process. Furthermore, the intensity threshold level for pixels to be considered in the fit can be freely adjusted, one can select between different methods for defining the IRF, and the pixel (ie, one of 128 pixels) based on which the IRF shall be defined can be freely chosen or will vary depending on the recorded FLIM data. Many of the choices available in SPCImage will result in a successful fit. For each and any of them it can be discussed extensively, whether the choice made is appropriate and the fit result might or might not be valid. Since such discussion will not be expedient, the FLIM images were analyzed by calculating the IRF at the brightest pixel and with the threshold set to auto.

The impact of different settings on the mean fluorescence lifetime of the same dataset was examined for a few samples, by changing the pixel for the IRF calculation, the bin factor and the threshold. The impact of different fitting algorithms has also been discussed literature [33].

Based on these thoughts and investigations, we strongly recommend to always include information about instrumentation and fitting procedures/parameters in publications to aid the generation and dissemination of valuable and reproducible fluorescence lifetime data.

4.2 | Suitability of coumarins with 4-hydroxy-TEMPO in ethanol as phantoms for NADH and FAD

Two-photon excitation at 780 nm is suitable for NADH and coumarin 1. The two-photon absorption cross section, expressed in units of Göppert-Mayer (1 GM = $10^{-50}$ cm$^{-4}$ s photon$^{-1}$ molecule$^{-1}$), is relatively small in case of NADH, with reported values for 780 nm of $\approx 0.003$ GM [34] and $\approx 0.02$ GM [35]. In comparison, for coumarin 1 in a concentration of $10^{-5}$ mol L$^{-1}$ in ethanol excited with a Ti:Sa laser, much higher two-photon absorption cross sections of 75.6 GM at 774.5 nm and 28.5 GM at 797.0 nm have been reported [10]. Correspondingly, rather low coumarin 1 concentrations had to be chosen to obtain fluorescence intensities similar to those of NADH typically observed in biological samples.

Above that, the following can be stated: The emission spectrum of coumarin 1 is narrower than that of NADH, as can be seen in Figure 2, but several important features are retained. The shapes of the emission spectra in the NADH bandpass filter range (436/20 nm) are similar, although the one of coumarin 1 is somewhat broader, and the long-wavelength tails of both emission spectra extend into the FAD bandpass filter range (562/40 nm), although at a considerably lower relative level in the case of coumarin 1. The fluorescence lifetime of the coumarin 1 phantom can be matched to that of NADH (ranging from 0.4 to 2.5 ns for free and bound NADH) by the quencher 4-hydroxy-TEMPO.

With this, it can be concluded that coumarin 1 fulfills all essential conditions to serve as a two-photon FLIM phantom for NADH: Its fluorescence can be excited at 780 nm and detected with a 436/20 nm bandpass filter, with fluorescence lifetimes matchable to those of NADH. The fluorescence of both fluorophores leaks into the employed FAD bandpass filter range.

For coumarin 6 the following can be stated: Two-photon excitation at 780 and 880 nm is possible for both FAD and coumarin 6. For coumarin 6, no information about two-photon absorption cross sections could be found in literature.

The emission spectrum of coumarin 6 largely resembles that of FAD, with similar widths and only a slight shift toward shorter wavelength in the case of coumarin 1, as can be seen in Figure 3. Both fluorophores show emission in the FAD bandpass filter range (562/40 nm), but not in the NADH bandpass filter range (436/20 nm). The fluorescence lifetime of coumarin 6 can be varied in the range of 0.2 to 2.5 ns, therefore the FAD phantom can at least be matched to the fluorescence lifetime of unbound FAD of $\approx 2.0$ ns. The quencher concentration necessary to reach the fluorescence lifetime of bound FAD of $\approx 0.1$ ns has to be tested. Alternatively, Rose Bengal (330 000, 95%, Sigma Aldrich) dissolved in water could be used to reach the short fluorescence lifetime of bound FAD. The fluorescence spectrum of coumarin 6 overlaps well with the range of the used bandpass for FAD detection and two-photon fluorescence excitation was well feasible with excitation wavelengths 780 and 880 nm. The obtained fluorescence lifetime is $\approx 0.1$ ns, which is consistent with literature values (0.12 ns [25]).
Thus, it can be concluded that coumarin 6 also fulfills all essential conditions to serve as a two-photon FLIM phantom for FAD: Its fluorescence can be excited at 780 and 880 nm and detected with a 562/40 nm bandpass filter, with fluorescence lifetimes matchable to those of unbound FAD and, at least approximately, also those of bound FAD. Furthermore, the fluorescence of both fluorophores does not leak into the employed NADH bandpass filter range.

Therefore both coumarin phantoms match the basic requirement for a phantom to have similar properties as the target fluorophores, but not necessarily identical ones [4]. Any other phantom features are, at this stage of phantom development, of negligible importance, but one has to be aware of their existence.

4.3 | Phantom limitations and development options

A major limitation of the proposed pair of liquid coumarin phantoms is that they can only be prepared to exhibit one single fluorescence lifetime. For example, a mixture of coexisting bound and free NADH (or FAD) cannot be mimicked with the proposed coumarin 1 (or coumarin 6) solutions with added 4-hydroxy-TEMPO, because it is not possible to have two different concentrations of the same quencher in one homogeneous solution with free convection. One alternative approach would be to use two different spectrally suitable fluorophores, provided that: (a) the two fluorophores are differently (but, coincidentally, exactly to the desired extent) affected by the same quencher or (b) a second spectrally suitable fluorophore/quencher system can be established in the same solution, such that the two fluorophore/quencher systems can be tuned more or less independently from each other. The fluorescence lifetime characteristics of mixtures of different fluorophores have been reported in literature, for example, for lucifer yellow/coumarin 6 and rhodamine B/coumarin 6 [12], but in both cases quenching was not investigated. For both mixtures, slight shifts in the fluorescence lifetimes of the components have been reported [12]. First experiments were carried out with a mixture of fluorescein in a 0.05 mol L\(^{-1}\) sulfuric acid solution of pH 3.5 and quinine sulfate quenched by sodium chloride (NaCl). The quenching of quinine sulfate worked, but the fluorescence lifetime of the fluorescein solution was found to increase by the addition of quinine sulfate and/or NaCl.

Finding the ideal combination between two fluorophores and two quenchers will require further research, to identify two fluorophores for which the same solvent can be used and two different quenchers are available who do not detrimentally affect each other or the other fluorophore/quencher system. In principle a simultaneous preparation of four different fluorescence lifetimes in the same solution would be desirable, to mimic coexisting bound and free variants of both NADH and FAD.

4.4 | Related intrinsic fluorophores

Inside living tissue there are further intrinsic fluorophores that coexist with NADH and FAD [36]. One fluorophore out of the flavin group is of particular interest: flavin mononucleotide (FMN). It has a similar fluorescence spectrum as FAD [37, 38] and will therefore, if present, unavoidably be detected by a bandpass filter tuned for FAD detection, so that a separation from FAD via emission wavelength is virtually impossible. Also, FMN is known by the authors to be similarly excitable as FAD. However, largely different two-photon excitation cross sections at 800 nm have been reported in literature: 2.1 GM for FAD and 1.6 GM for FMN in one source [37], as well as \(\approx 0.034\) GM for FAD [34] and \(\approx 0.17\) GM for FMN [39] in two other sources (data in both cases extracted using a graph digitizer [40]).

The potential contribution of FMN to measurements in cell cultures and in vivo is usually not taken into account so far. Apparently, a distinction from FAD is almost impossible based on spectral characteristics of the two molecules, but might be possible based on fluorescence lifetimes [41]. As a potential phantom for FMN, lucifer yellow could be suitable, with a fluorescence lifetime in water of \(\tau \approx 5.2\) and \(\tau \approx 10\) ns in ethanol [12]. If a suitable quencher can be identified, lucifer yellow could potentially act as phantom for FMN.

5 | CONCLUSION

In conclusion a reproducible and stable standard phantom for two-photon FLIM can be established according to the mentioned recipes, mimicking FAD and NADH in their bound and unbound states. By means of these phantoms, measurements of different two-photon FLIM systems can be made more comparable since the inherent unstable characteristics of NADH and FAD are circumvented. Further investigations would be necessary to create a structured standard with sufficiently small features, which could then be useful to improve the localization of two-photon FLIM signals in samples.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT
Research data are not shared.

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