Intensity-independent fluorometric detection of cellular nitric oxide release

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Abstract A new fluorescence method is introduced in which nitric oxide (NO)-derived higher-order oxygen complexes (NO_x) are quantified at physiological pH. Detecting the fluorescence lifetime shift between 2,3-diaminonaphthalene and the NO_x-derived protonated 2,3-naphthotriazole allows an intensity independent determination of the NO_x concentration. The NO release from LPS and IFNγ-stimulated murine macrophages and iNOS transfected hamster cells was quantified. The lower detection limit for NO_2^- was found to be 800 pmol/ml. Since the influence of static fluorescence quenching due to cellular components can be neglected, the method is applicable for clear cellular supernatants as well as turbid cellular suspensions.

Key words: Nitric oxide; Fluorescence spectroscopy; Diaminonaphthalene; Single-photon counting

1. Introduction

The paramagnetic radical nitric oxide (NO) plays a critical role in cell signalling and is a key player in the pathophysiology of several diseases [1,2]. It was first recognised by Furchgott and Zawadzki [3] that endogenous vasodilators such as acetylcholine act on endothelial cells rather than directly on vascular smooth muscle, triggering the release of a labile factor which is able to diffuse to the neighbouring smooth muscle. It is now generally accepted that this endothelium-derived relaxing factor is NO [4,5] which activates guanylate cyclase with the subsequent accumulation of cyclic guanosine monophosphate leading to vasodilation. Elevated NO levels and their action on guanylate cyclase in vascular smooth muscle is the reason for the widespread use of exogenously added organic nitrates (e.g. nitroglycerin and nitroprusside) in medicine since the last century as antianginal and antihypertensive agents [6].

NO is synthesised from the guanidino nitrogen of L-arginine by at least two distinct isoforms of the enzyme nitric oxide synthase (NOS). Constitutively expressed NOS isoforms (cNOS) are activated by an increase in intracellular Ca^{2+} levels, and hence generally produce short pulses of NO. NO produced by endothelial cNOS mediates blood vessel dilatation, as described above. In the central and peripheral nervous systems, NO is produced by another Ca^{2+}-regulated isoform of NOS termed bNOS or nNOS, and is involved in neural signalling [7]. A second isoform of NOS, termed iNOS, is expressed upon stimulation with cytokines and bacterial products such as lipopolysaccharide (LPS) [8]. Both the iNOS and cNOS isoforms require NADPH, FMN, FAD, and tetrahydrobiopterin (THB) as cofactors or prosthetic groups. However, in contrast to cNOS, iNOS is not dependent on high Ca^{2+} levels for activity, and once expressed, is capable of producing large amounts of NO over several hours or even days. Many different cell types are capable of expressing iNOS, including endothelial cells, fibroblasts, and hepatocytes, although macrophages are among the best characterised iNOS-expressing cells [8].

Depending on the concentration and microenvironment, NO can rapidly and spontaneously react with molecular oxygen to yield a variety of nitrogen oxides, including nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3) and nitrite (NO_2^-). NO_2 and N_2O_3 were shown to be potent oxidizing and N-nitrosating agents [9], although the exact nature of the specific NO-derived N-nitrosating agent(s) is still unknown. This N-nitrosating characteristic of NO_x derived from NO is frequently used for its determination. In fluorescence assays, the aromatic diamino compound 2,3-diaminonaphthalene (DAN) has been used as an indicator of NO formation and release [10]. However, the sensitivity of these methods relies on a strong alkalinisation of the sample, thereby generating the 2,3-naphthotriazole anion which has a much higher quantum yield than DAN itself [11]. The resulting amplified fluorescence intensity signal is used to quantify NO_x formation. We present here a novel fluorescence method, the principle of which does not depend on fluorescence intensity changes but on fluorescence lifetime shifts of the DAN fluorophore. The detection limit for the basic fluorescence emission of DAN is much lower than the linear correlation of lifetime shifts and NO_x concentration. Furthermore, since experiments are accomplished at physiological pH and therefore avoid strong alkaline conditions, the method is applicable to detection of NO release from living cells.

2. Material and methods

2.1. Cell culture and induction of NOS activity

The murine macrophage cell line, RAW 264.7 (American Type Culture Collection, Rockville, MD), was cultured in DMEM H21 containing 10% fetal calf serum, 10 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, at 37°C in a humidified atmosphere of 5% CO_2. V79 Chinese hamster cells stably expressing human iNOS were cultured in DMEM H21, as described above, with additional

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Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; NO_x, nitric oxides; NO_2^-, nitrite; DAN, 2,3-diaminonaphthalene; NATH, 2,3-naphthotriazole (protonated); LPS, lipopolysaccharide; IFNγ, interferon-γ; SPC, single-photon counting

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supplementation of the antibiotic G418 (Gibco BRL, Grand Island, NY).

For induction of NOS activity, confluent monolayers of RAW 264.7 cells were stimulated with 10 ng/ml LPS from *Salmonella abortus equi* (Sigma Chemicals Co., St. Louis, MO) and 10 U/ml murine IFNγ (Genzyme Corporation, Cambridge, MA) for 8–14 h, after which the cells were rinsed with PBS and harvested by scraping into PBS with a rubber policeman. The supernatant was retained for analysis of nitrite using the Griess assay [12]. The cells were centrifuged at 1000 rpm and resuspended in the assay medium (RPMI without phenol red, supplemented with 20 mM HEPES, 1.14 mM L-arginine (Gibco BRL, Grand Island, NY) and 200 μM DAN (Fluka, Buchs, Switzerland) in 2% DMF)) at the indicated cell density. The cells were seeded into 12-well plates (Costar, Cambridge, MA) at 1 ml/well, and incubated for the indicated time. The supernatant was then centrifuged to remove any contaminating cells and stored in the dark on ice until measurement.

V79 hamster cells were incubated in the assay medium as described for RAW 264.7 cells, at a concentration of 2×10^6 cells/ml.

2.2. Steady state fluorescence measurements

Steady state fluorescence measurements were performed on a SLM 8000C (SLM Instruments, Urbana, IL). Fluorescence excitation spectra were corrected for the wavelength characteristics of the xenon lamp by the ratio mode method, and fluorescence emission spectra were corrected for buffer background emission. Commonly, slit widths giving a bandwidth of 2 nm were used. The integration time for 1 nm steps was 1 s in a 1 cm path length cuvette. All fluorescence measurements were performed with polarisers in magic angle setting which reduces the influence of stray light especially in cellular experiments.

2.3. Picosecond time-resolved fluorescence measurements

Picosecond time-resolved fluorescence measurements were recorded according to the single-photon counting (SPC) technique [13]. An Ar^+-pumped Ti:Sapphire laser (model MIRA 900, Coherent, Santa Clara, CA) provided ultrashort pulses under mode-locked conditions (FWHM = 150 fs) with a frequency of 75 MHz. To reduce the repetition rate to 4.7 MHz, a pulse picker (model 9200, Coherent, Santa Clara, CA) was inserted into the beam. The beam was then split to (i) trigger a fast photo diode, which yields after discrimination (Ortec pico-timing discriminator 9307, Oak Ridge, TN) the stop signal for the SPC measurements (reverse mode), and (ii) to excite the sample. The excitation wavelength of 360 nm was obtained by first manually tuning the MIRA 900 to 720 nm and then doubling the frequency of the beam with the ultrafast harmonic generation system 5-050 (Inrad, Northvale, USA). The resulting excitation power ranged between 0.1 and 0.3 mW, the full width at half maximum of the instrument response function being 70–80 ps. Fluorescence photons were collected at 425 nm in a Spex 1681 single grating monochromator (Spex, Edison, USA) and detected with the Hamamatsu R3809U microchannel plate (Hamamatsu, Shimokanzo, Japan). This signal was preamplified (preamplifier 9306, Ortec, Oak Ridge, TN), and discriminated (discriminator 9307, Ortec, Oak Ridge, TN) before it was used to start the voltage ramp in the time-to-amplitude converter, which was stopped in the reverse SPC mode by the next incoming signal of the photodiode. The voltage values which correspond to the time passed between excitation and emission of a fluorescence photon in a single experiment were stored in the Spectrum Master 921 (Ortec, Oak Ridge, TN) which was connected to the multichannel emulation programme Maestro (Ortec, Oak Ridge, TN). SPC raw data were iteratively reconvoluted with the FLA900 programme from Edinburgh Instruments (Edinburgh, UK) according to the multiexponential model

$$F(t) = A + \sum b_i \exp(-(t+\delta t)/\tau_i) \quad (1)$$

where A represents the background noise of the experiment, b_i represent the pre-exponential terms of the according fluorescence lifetimes τ_i, and δt is an optional temporal shift term to compensate for shifts of the instrument response. Relative amplitudes B_i are obtained by computing

$$B_i = [(b_i\tau_i)/\sum(b_i\tau_i)]\times100(\%) \quad (2)$$

3. Results and discussion

Curve A in Fig. 1 shows the emission spectrum of 200 μM DAN in PBS (pH 7.2) upon excitation at the maximum absorption wavelength of 360 nm. Maximum emission was obtained at 394 nm, which corresponds to the expected small Stoke's shift of DAN in a pH-neutral medium. After incubating 200 μM DAN with 100 μM of the spontaneously NO-releasing compound S-nitroso-*N*-acetylpenicillamine for 1 h, the emission spectrum changes (curve B, Fig. 1). The short-wavelength shoulder of the DAN spectrum at 375 nm decreases, whereas a tail at longer wavelengths appears. Moreover, the emission maximum shifts to 398 nm. The primary product of the acid-catalysed nitrosation reaction of DAN is

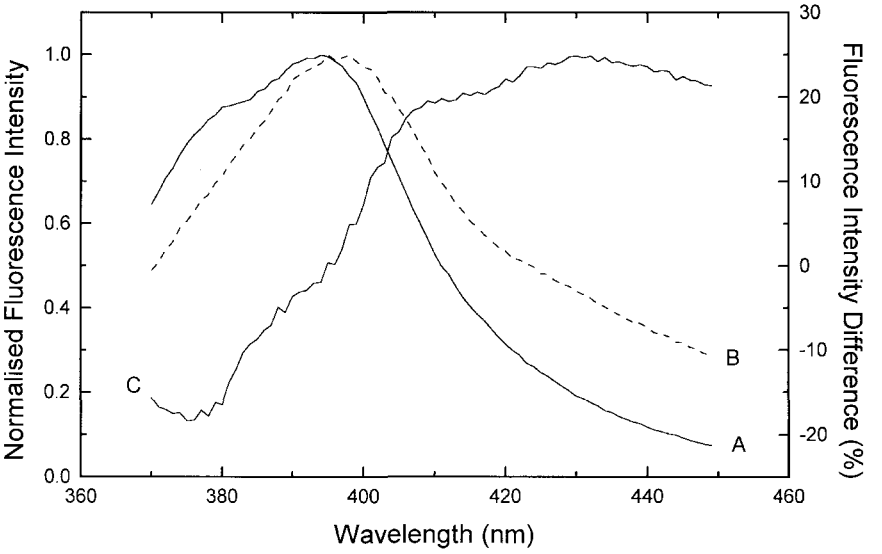

Fig. 1. Fluorescence emission spectra of DAN and NATH. Curve A (solid line): Normalised fluorescence emission spectra of 200 μM DAN in PBS (2% DMF, 1% methanol). Curve B (dashed line): 200 μM DAN incubated for 1 h with 100 μM S-nitroso-*N*-acetylpenicillamine in PBS (2% DMF, 1% methanol). The presented spectra were recorded with the excitation wavelength set at 365 nm and were corrected for background emission. Curve C: Fluorescence difference spectrum B−A.

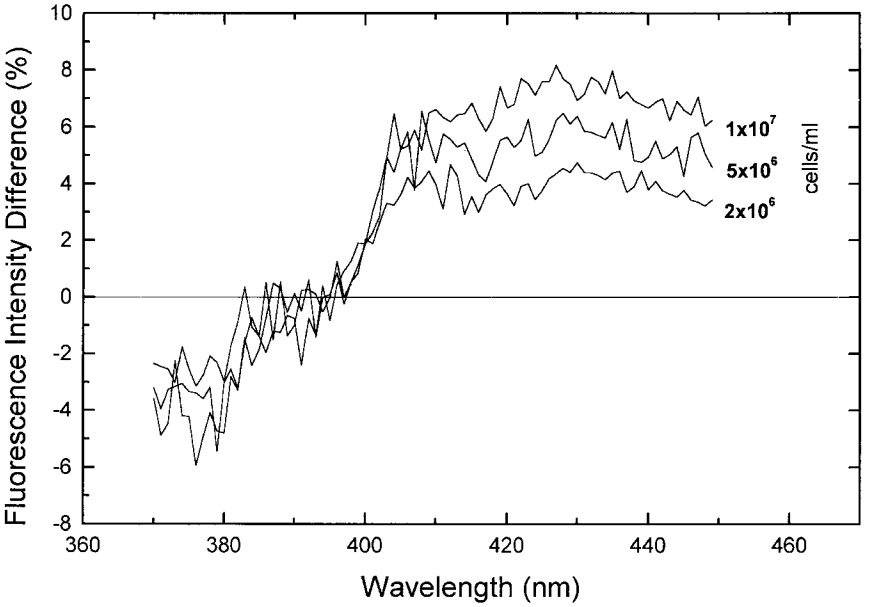

Fig. 2. Effect of NO produced by macrophages on the fluorescence emission of DAN. Fluorescence difference spectra of the normalised emission of 200 μM DAN, incubated with different numbers of stimulated RAW 264.7 cells, minus the normalised emission of 200 μM DAN, incubated with the respective number of unstimulated RAW 264.7 cells. The incubation time was in every case 1 h.

2,3-naphthotriazole [14]. In our neutral reaction mixture we did, however, not observe the frequently published alkaline emission spectrum of the triazole, which exhibits maxima at 395, 415 and 455 nm (compare Fig. 1 in this study with Fig. 6 in [15]). Only under strong alkaline conditions (pH > 12) were we able to detect this spectral pattern. We therefore conclude that the single broad emission band obtained at pH = 7.2 is most probably due to the protonated 2,3-naphthotriazole, NATH. The emission spectra seen in the literature are the result of generating the naphthotriazole anion in strong alkaline media, which characteristically exhibit enhanced fluorescence emission due to reduced intersystem crossing quenching. For matters of clarity we will refer in the following to the protonated form of the 2,3-naphthotriazole as NATH. The fluorescence difference spectrum F(NATH)-F(DAN) is shown as curve C in Fig. 1.

The spectral difference itself between DAN and NATH provides an easy-to-use method for detecting NO released from murine macrophages. Fluorescence difference spectra reveal the relative change in fluorescence intensity upon incubating DAN (200 μM) with NO released from stimulated RAW 264.7 cells, as is shown in Fig. 2. Increasing the cell number, and therefore the amount of NO released into the medium, increases the fluorescence difference band at 425 nm accordingly. However, the changes in the relative fluorescence difference band are not very high compared to the sensitivity of the method itself: a 8 ± 1% difference band is obtained when 1×10^7 cells are used, which corresponds to a 12.5% relative error. Therefore we extended the method using the temporal behaviour of the DAN fluorescence emission with the aim of gaining sensitivity in a physiological environment (i.e. at neutral pH). The new method relies not on changes in intensity but on changes in the fluorescence transients.

The fluorescence decay of DAN in the assay medium, i.e. in the supernatant of unstimulated RAW 264.7 cells (see Section 2), is best described by three exponential terms (see Table 1). The very short lifetime $\tau_1 < 100$ ps is due to a small contamination ($B_1 < 1\%$) of laser scatter which does not interfere with data analysis. $\tau_2 = 0.9$ ns refers to a photophysical state of DAN with very low population in the excited state ($B_2 < 6\%$). It is likely that a tautomeric isoform of DAN which is conformationally unstable under these solvent conditions is responsible for this photophysical state. The main lifetime component is, however, $\tau_3 = 6.12$ ns with an amplitude $B_3 > 90\%$, both of which remained constant during a time-resolved measurement. This is a rather short lifetime compared to 1-aminonaphthalene, which decays in ethanol with 19.6 ns [16]. The quenching of DAN in aqueous solutions

Table 1
Parameters of the time-resolved fluorescence spectra analysis

Cell no.	Incubation (min)	B_1 (%)	τ_1 (ns)	B_2 (%)	τ_2 (ns)	B_3 (%)	τ_3 (ns)	χ^2
2×10^6	60	0.79	0.106	5.56	1.239	93.64	6.931	1.23
5×10^6	60	0.82	0.109	5.48	1.278	93.69	7.319	1.15
1×10^7	60	0.89	0.144	5.89	1.384	93.22	7.983	1.21
1×10^7	20	0.91	0.071	5.21	1.113	93.88	6.81	1.25
1×10^7	40	0.89	0.090	5.35	1.217	93.76	7.350	1.32
1×10^7 unstim.	60	0.97	0.063	5.19	0.908	93.84	6.129	1.31

Best-fit reconvolution parameters of time-resolved fluorescence spectra of 200 TM DAN incubated with different numbers of (un)stimulated RAW 264.7 cells with different incubation times according to Eqs. 1 and 2.

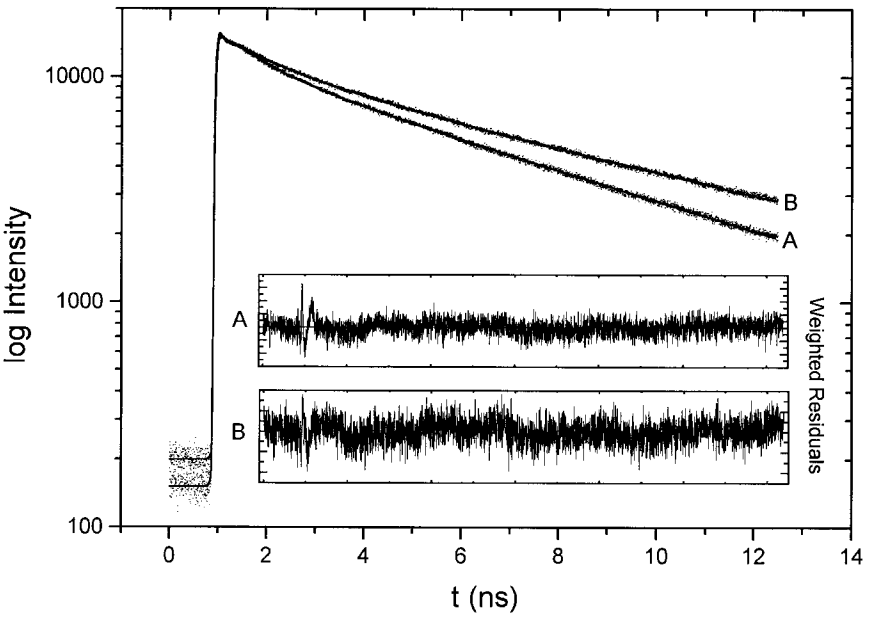

Fig. 3. Effect of NO produced by macrophages on the fluorescence transient of DAN. Time resolved fluorescence spectra of 200 TM DAN incubated with 1×10^7 unstimulated (A) or stimulated (B) RAW 264.7 cells. The incubation time was in both cases 1 h. The time-resolved fluorescence raw data (dots) are shown together with the best fit (solid line) according to Eq. 1. The weighted residuals for (A) and (B) are presented in the inserts.

(containing 2% DMF) can be attributed to proton transfer and collisions with solvent molecules, thereby reducing the natural lifetime of the chromophore.

After incubation of DAN with stimulated cells (see Section 2), a clear increase in the value of τ_3 is observed whereas the amplitude B_3 remained constant (see Table 1). The magnitude of increase depends upon cell number and incubation time. To illustrate this effect the time-resolved raw data plus best iterative reconvolutive fit are shown in Fig. 3 for 1×10^7 unstimulated (curve A) and for 1×10^7 stimulated (curve B) RAW 264.7 cells. The incubation time with DAN was in each case 60 min. Pelleting the cells by centrifugation and investigating the supernatant only provided a means for defining clear time points for DAN and NO incubation without continuing the reaction during measurements. By this means, we were able to show that an incubation time of 20 min is sufficient for reliable detection of NO released from cultured cells (see Table 1).

The increase of τ_3 is the result of N-nitrosation of one amino group of DAN due to the NO released from the cells.

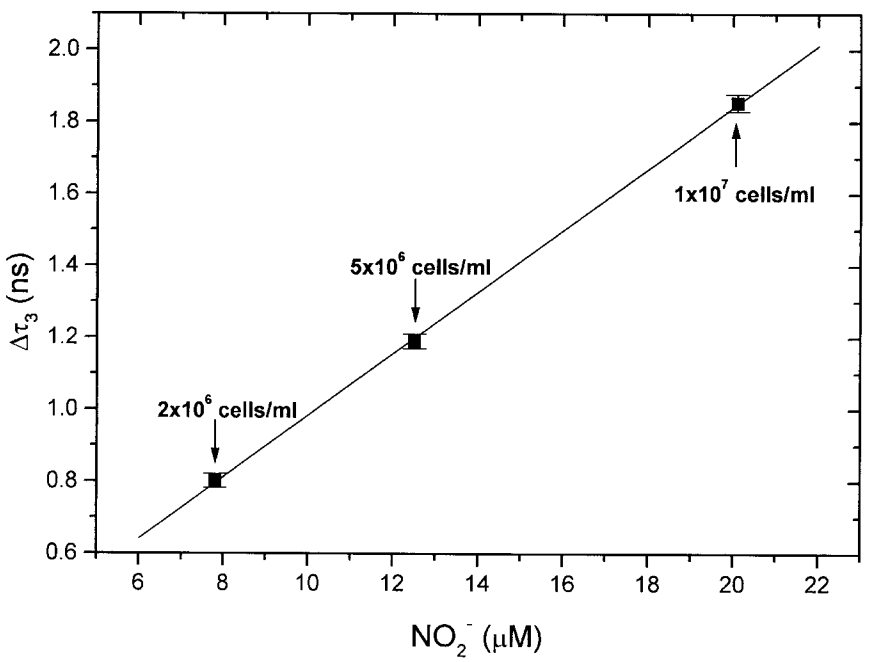

Fig. 4. Fluorescence lifetime shift $\Delta\tau_3$ as an indicator for NO produced by macrophages. Correlation of fluorescence lifetime shift $\Delta\tau_3$ with the amount of nitrite produced by increasing numbers of stimulated RAW 264.7 cells. The correlation was $R=0.99989$.

A similar increase has been found for the reaction of DAN with the NO-releasing compound SNAP (data not shown). Since the increase of τ_3 is independent of the fluorescence intensity, a relatively weakly fluorescing compound like DAN can be used to quantify NO release via NATH generation, without requiring strong pH changes to obtain the much better fluorescing triazole anion [10]. The lower limit for DAN and NATH fluorescence detection is >1 nM.

Fig. 4 shows the linear dependence of the increase of τ_3 upon the nitrite concentration in the supernatants of different numbers of stimulated RAW 264.7 cells, expressed as lifetime shift $\Delta\tau_3$. A standard curve with a high correlation coefficient $R=0.99989$ for NO_2^- detection in the μM range (nmol/ml) was obtained spectrophotometrically using the Griess assay [12]. This correlation between $\Delta\tau_3$ and NO_2^- production was used to determine NO_2^- levels produced by V79 hamster cells stably transfected with the *iNOS* gene. The cells, which constitutively produce NO, were incubated at 2×10^6/ml for 60 min in the DAN assay medium. The time-resolved fluorescence decay was then measured for 20 min directly in the stirred cellular suspension, without prior centrifugation. The amount of NO_2^- thus determined was 3 μM. No change of τ_3 during the period of measurement (20 min) was detected. This means that a steady state between NO_x generation and DAN nitrosation was reached after 60 min incubation time and that the $\Delta\tau_3$ measured is indeed directly proportional to the NO release under these conditions. To explore the limits of the method, different concentrations of $NaNO_2$ were incubated with 200 μM DAN under the same conditions as the incubations with cell supernatants (see Section 2). A significant lifetime shift $\Delta\tau_3>200$ ps was obtained at concentrations $\geq$800 nM (data not shown).

With the method presented here the release of micromolar amounts of NO under physiological conditions can be detected. In the future, NO generation in other cell types shall be investigated by fluorescence lifetime imaging [17]. Strong acidic or alkaline conditions as used in the common fluorescence assays [15] should therefore be avoided. In addition, since our method does not depend upon changes in fluorescence intensity, ground state quenching of the DAN/NATH emission, which certainly does occur in cells, will not disturb the interpretation of lifetime shift results recorded in vivo. The use of other aromatic *o*-diamino compounds with a higher fluorescence quantum yield than DAN should furthermore extend the detection limit for nitrite to the low nM range.

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