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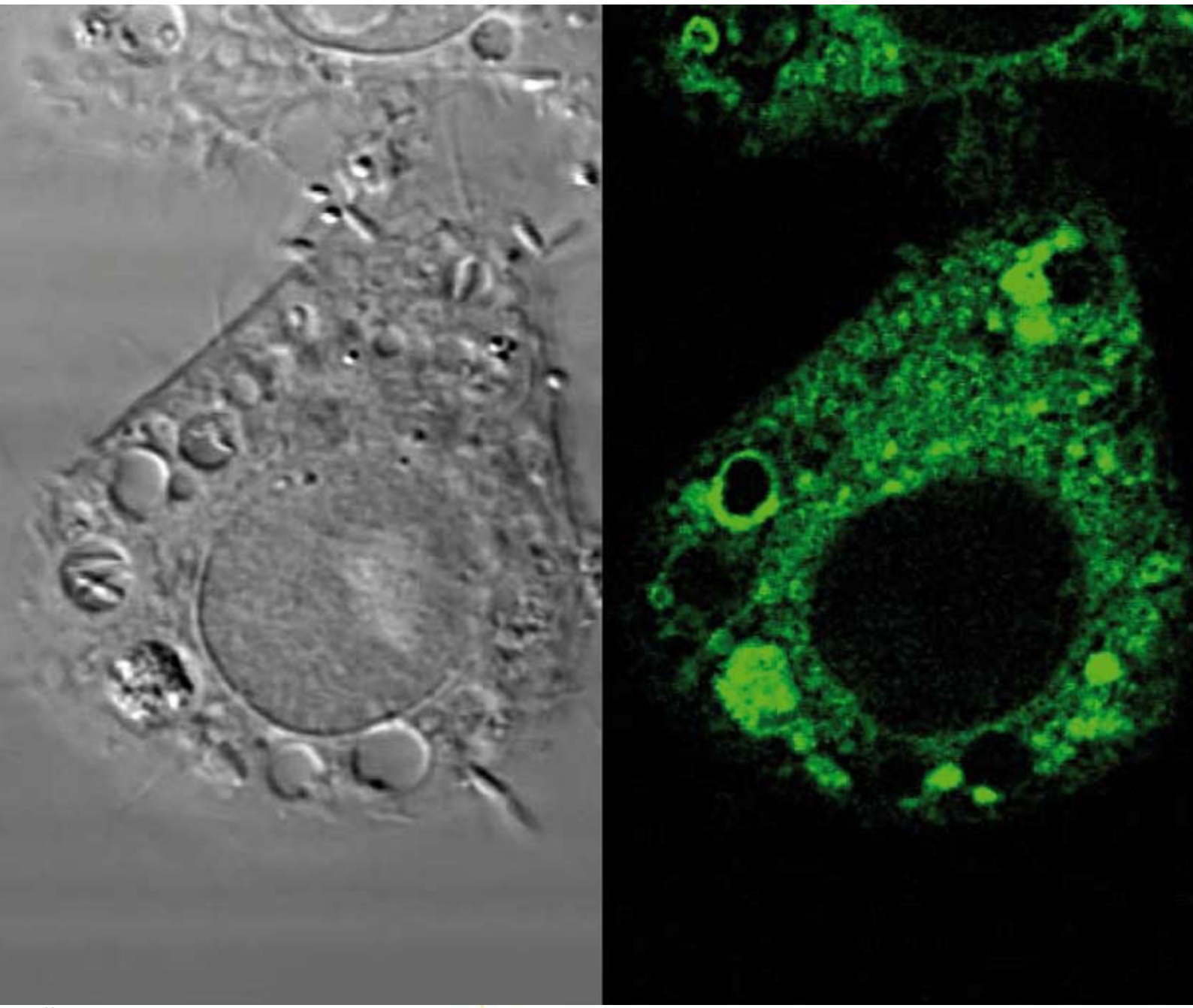

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Spectroscopic studies of 1,2-diaminoanthraquinone (DAQ) as a fluorescent probe for the imaging of nitric oxide in living cells

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Spectroscopic analysis of the fluorescent probe 1,2-diaminoanthraquinone (**DAQ**) provides information about the mechanism of nitric oxide imaging in living cells. Fluorescent aggregates of a reaction product of **DAQ** are thought to be responsible for the images obtained with confocal fluorescence microscopy.

Nitric oxide (NO) is a free radical that has been the subject of intense research[1] following its implication in numerous biological functions.[2,3] NO acts as a vasodilator in the cardiovascular system,[4] as a neurotransmitter,[5] and as effector within the immune system.[6] Specific probes for imaging NO in living cells or tissues have been designed and commercialised, which have accelerated rapidly the research in the field. Fluorescence represents one of the most useful tools (together with EPR) to visualise the presence of NO in biological systems[7–10] and recent reviews can be found in the literature.[11–15] One of the probes commercialised for NO sensing is 1,2-diaminoanthraquinone (**DAQ** or **DAA**) (Chart 1). It has been reported that **DAQ** is not cytotoxic,[16] in contrast with other NO probes, and it does not need ester derivatives for cellular uptake.[16] It has been reported that the non-fluorescent **DAQ** reacts with NO in the presence of oxygen to give the triazole **DAQ-TZ** (Chart 1) which is detectable by means of fluorescence microscopy.[16] Further experiments reproduced this observation.[17–22] However, in the course of our research on nitric oxide detection systems[23–25] we found some apparent inconsistencies about the use of **DAQ** for the detection of NO. Accordingly we decided to undertake a spectroscopic study of this probe in solution and intracellularly in order to establish the species responsible for the fluorescence observed in the biological samples.

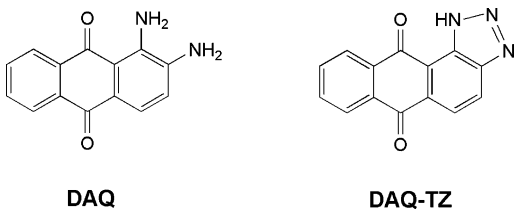

Chart 1

The original description of the reaction between **DAQ** and NO was reported by Heiduschka and Thanos:[16] a glass slide containing an aqueous solution of **DAQ** was exposed to commercially available NO gas. The colour of **DAQ** changed from violet to brownish and a new *red fluorescence* became visible with a rhodamine filter (above 580 nm). It was reported that the triazole formed was water insoluble and that it precipitated. Similar experiments were performed by us by preparing two air equilibrated solutions: (a) **DAQ** 50 μM in water : DMSO (9 : 1), pH 7.4, HEPES 10 mM, NaCl 0.1 M, and (b) **DAQ** 50 μM in DMSO. Pure NO gas (3 ml) was bubbled slowly into each solution. Such a quantity of gas was enough to obtain a saturated solution of NO.[11] In both cases the rapid bleaching of the initial pink-red colour to yield a colourless solution was observed as shown in the inset of Fig. 1(a). However, no visible precipitate could be seen with the concentrations used.

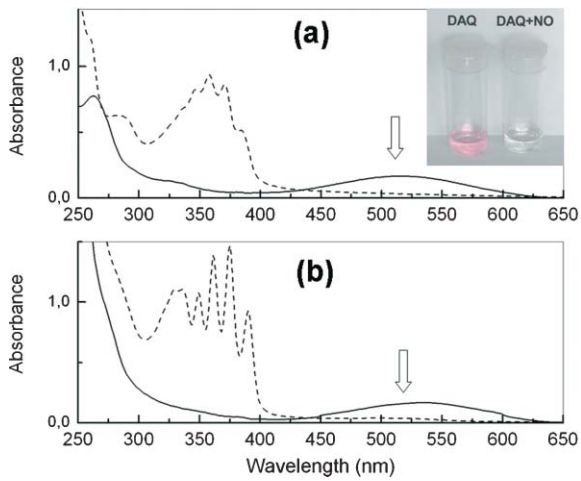

Fig. 1 Absorption spectra of **DAQ** before (——) and after reaction (---) with gaseous NO. (a) **DAQ** 50 μM in water : DMSO (9 : 1) pH 7.4. (b) **DAQ** 50 μM in DMSO.

In the absorption spectra, the elimination of the band at *ca.* 450–600 nm in both solutions (a and b) was observed after 15 min (Fig. 1). The same result was also observed in water–DMSO solution at pH 5.9 (data not shown). These absorption changes can be interpreted in terms of reaction of the electron pairs of the free amino groups of **DAQ** to form the **DAQ-TZ** triazole (hence eliminating the CT band) responsible for the red colour.[26] In fact, the addition of a few drops of concentrated HCl to a solution of **DAQ** induces bleaching of the red colour due to the protonation of the amino groups.

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On the other hand, the weak fluorescence emission of the solutions of **DAQ**[27] was also quenched following reaction with gaseous NO (in oxygenated medium). These observations raised some questions about the use of **DAQ** for fluorescence microscopy, since previous reports rely on the excitation of a band (centred at *ca.* 520 nm) that no longer exists when NO is present. It is also apparent that the resulting NO product (**DAQ-TZ**) does not absorb at wavelengths reported for excitation of **DAQ** in cells or tissues (>520 nm).

Imaging of NO by means of **DAQ** in biological samples has been extensively reported and accordingly we attempted the visualization of NO in living cells. Thus, a culture of Raw 264.7 mouse macrophage cells was loaded with **DAQ** and stimulated to produce NO following standard procedures[28] and examined by means of confocal laser fluorescence microscopy. Excitation of the intracellularly loaded **DAQ** with both $\lambda = 543$ and 488 nm gave the results shown in Fig. 2, the excitation of the **DAQ** at 488 nm affording a brighter image than at 543 nm. The nature of the high fluorescence intensity positive areas is currently being investigated. However, co-localization experiments with the pH probe FG-H503[29] suggest a lysosomal identity, and also an important role of acidic pH. No subcellular structures could be visualized using **DAQ** in non-stimulated cells.

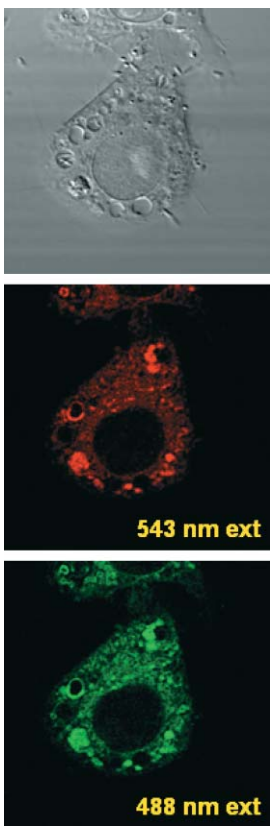

Fig. 2 Confocal laser fluorescence microscopy image of Raw 264.7 cells loaded with **DAQ** and stimulated to produce nitric oxide. Images correspond to DIC channel (top), 543 nm and 488 nm excitation.

To address the question of how the biological experiments exciting at >520 nm could afford fluorescence from **DAQ** if no absorption is apparent (since **DAQ** absorption is quenched by NO), a working hypothesis was considered: the conditions of bioimaging are such that a high concentration of **DAQ-TZ** molecules occurs and that supramolecular aggregates of **DAQ-TZ** are formed which are able to emit fluorescence. As previously discussed, the original paper[16] reported that a brownish precipitate appeared upon exposure of **DAQ** to NO gas, which supports the possible formation of aggregates. In this regard, it should be noted that the formation of fluorescent aggregates is a common photophysical phenomenon and many examples of aggregation-induced emission can be found in the literature.[30]

In order to confirm this hypothesis the fluorescence of three solutions containing **DAQ** at varying concentrations, in DMSO (5, 50 and 500 μM) were studied. To 1 ml of such solutions (air equilibrated), 6 ml of NO gas was bubbled. For the 5 μM and 50 μM **DAQ** the initial pink-red colour disappeared within a few seconds to yield colourless solutions. With the 500 μM **DAQ** solution a light brown solution was obtained after addition of the NO gas. The fluorescence of these solutions showed, upon increasing the concentration, a relative rise of the shoulder at *ca.* 600 nm relative to the band at *ca.* 440. The fluorescence emission spectra of these three solutions were recorded exciting at 350 nm and the results can be seen in Fig. 3(a). With the dilute solution (5 μM) the emission spectrum exhibited a maximum at 440 nm only. However at 500 μM the **DAQ** solution showed two well distinguished emission bands at *ca.* 450 nm and 580 nm.

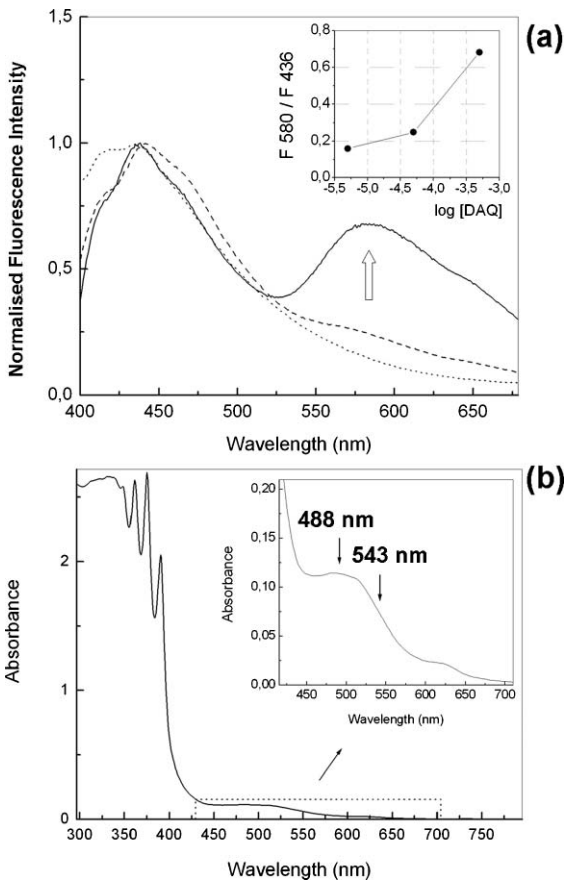

Fig. 3 (a) Fluorescence spectra after addition of NO gas to varying concentrations of **DAQ** in DMSO: 5 μM (···), 50 μM (---), 500 μM (——). Excitation wavelength = 350 nm. Inset: intensity ratios at 580 and 436 nm *vs.* **DAQ** concentration. (b) Absorption spectrum of **DAQ** (500 μM) in DMSO after reaction with gaseous NO.

These bands can be tentatively assigned to the emission of the monomer and aggregate of the triazole **DAQ-TZ**, respectively. The relationship between the monomer/aggregate intensity ratios and probe concentration can be seen in the inset of Fig. 3(a). If no aggregation process had taking place a horizontal line would be expected. However it is clear from Fig. 3(a) (inset) that the ratio of fluorescence emission intensities (F580/F436) increases with increasing DAQ concentration.

A careful inspection of the absorption spectrum of the brownish solution (not precipitated) in DMSO after reaction with NO (500 μM in DAQ) afforded some insight. As it can be seen in Fig. 3(b), there is a discernable absorption between 450 and 650 nm which could be attributed to the putative aggregates, which also would impart the brown colour to the DMSO solution.

In another experiment the solvent was changed to tetrahydrofuran (THF) so that the **DAQ-TZ** aggregates would be soluble enough, as in DMSO, to enable the detection of the fluorescence signal. Analogously, the fluorescence intensity ratios increased with increasing concentration of **DAQ** between 5 and 180 μM (Fig. 4(a)). Additionally the absorption spectra of such solutions showed a new absorption band at *ca.* 450–500 nm (Fig. 4(b)) which did not follow the linearity predicted by the Beer law (inset of Fig. 4(b)).

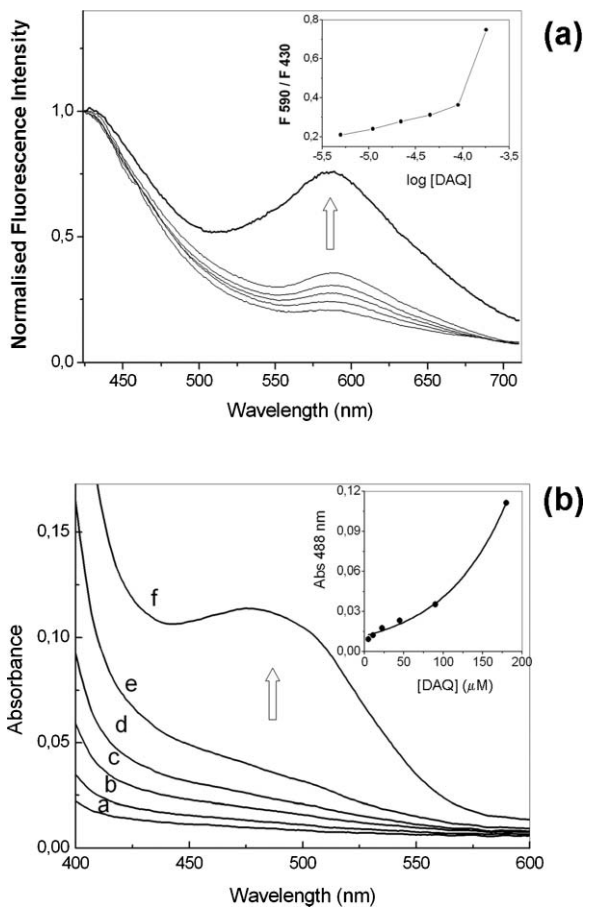

Fig. 4 (a) Fluorescence spectra after addition of NO gas to varying concentrations of **DAQ** in THF (5–180 μM). Excitation wavelength = 350 nm. Inset: intensity ratios at 590 and 430 nm *vs.* **DAQ** concentration. (b) Absorption spectrum of **DAQ** in THF (a = 5, b = 10, c = 20, d = 45, e = 90, f = 180 μM) after reaction with gaseous NO. Inset: absorbance intensity at 488 nm *vs.* concentration of probe.

A comparison of the recorded fluorescence spectra obtained with three typical wavelengths used in confocal fluorescence microscopy is shown in Fig. 5. As it can be seen, 488 nm excitation yields greater emission intensity than that of both 543 nm and 364 nm, in agreement with the higher absorption at such wavelength.

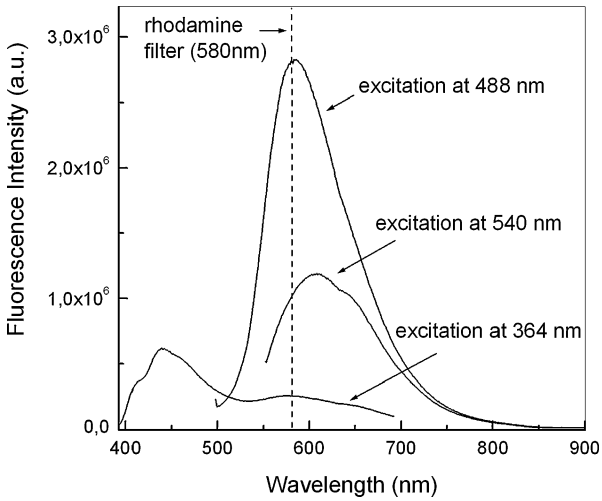

Fig. 5 Effect of excitation wavelength on the fluorescence. Addition of NO gas to **DAQ** (500 μM) in DMSO. The cut-off of the rhodamine filter is shown to illustrate the emission eliminated with its use.

The main *practical consequence* of the above findings is that knowing the species origin of the emission it is possible not only to excite the fluorophore selectively but also to collect the maximum amount of light from the sample through selection of suitable emission filters. In the original application of **DAQ**[16] it is reported that a rhodamine filter should be used (cutting off 580 nm and below). Moreover in subsequent reports the specific use of 520 nm[19] and 543 nm[17] excitation was described. However, with consideration of the results reported here *it would seem that a better choice would be to excite the samples at a shorter wavelength value and collect the emission even below the rhodamine filter (580 nm)*. Excitation at the wavelength of the argon-ion laser, typically used in confocal laser scanning microscopy (488 nm), rather than that of the helium–neon laser (543 nm), and collecting the emitted light above 500 nm, would improve the imaging experiments. This fact would explain, in part, the bright image recorded with excitation at 488 nm, in comparison with the one using 543 nm.

In addition to this practical application for improved imaging of NO, a mechanistic implication can be also inferred. Oxygenated solutions of nitric oxide react with **DAQ** but the product is only detected when the concentration of both are high enough to yield fluorescent aggregates of **DAQ-TZ**. Consequently those cellular regions not showing fluorescence emission may contain some NO but at lower concentrations. Other probes sensitive to NO should be used complementarily.[7,11–15] We are aware of the complexities of the intracellular world and hence we can not rule out any secondary reaction with **DAQ** leading to a fluorescent product different from **DAQ-TZ** in the cells.[31] However, this seems

unlikely as NO inhibitors have been used,[17–19] demonstrating that the fluorescent signal correlates well with the presence of NO.

In summary, the spectroscopic analysis of **DAQ** and **DAQ-TZ** has provided some insights into the mechanism of NO imaging in live cells. With consideration that (a) **DAQ** is strongly coloured but practically non-fluorescent, (b) that **DAQ-TZ** is colourless in dilute solutions (not able to absorb appreciably at >450 nm) and (c) that as **DAQ-TZ** tends to associate in DMSO and THF it is thought that the aggregates of **DAQ-TZ** (precipitated into the cells) are the possible origin for the fluorescent images acquired by microscopy, but not the isolated **DAQ-TZ**. A practical implication of this result is apparent: it is optimal to excite at the maximum absorption of the aggregates, *i.e.*, 488 nm.

Experimental details: **DAQ** and other reagents were purchased from Sigma-Aldrich. Solvents were of analytical reagent grade or better. Water used was Millipore® quality. UV-vis spectra were recorded using a Hitachi U-3000 spectrophotometer. Steady state emission and excitation spectra were recorded using a FluoroMax-2 instrument. 1 cm path-length quartz cells were used for both the absorption and emission measurements. All the solutions were air-equilibrated prior to reaction with NO (gas). For all the experiments with NO, saturating conditions were obtained in order to attain complete conversion of **DAQ** to **DAQ-TZ**. Therefore the corresponding **DAQ** solution in aqueous or organic solvent was placed in the quartz cell and after measurement of absorption and fluorescence spectra, an excess of NO (gas) was bubbled slowly through the sample (typically 3–6 ml injected by means of a syringe). After a sufficient stabilization period, typically a few minutes, the absorption and fluorescence spectra were again recorded. The macrophage cells were imaged using a confocal laser scanning microscope (Carl Zeiss, LSM 510 meta) with 543 nm and 488 nm laser excitation. Appropriate emission bands were selected for the two fluorescent channels. A 63×, 1.4 NA objective was used to ensure high-resolution images. Details of cell growth have been reported previously.[29]

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