# DEVELOPMENT OF A FLUORESCENT INDICATOR FOR NITRIC OXIDE BASED ON THE FLUORESCEIN CHROMOPHORE

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Endogenous nitric oxide (NO) appears to modulate many physiological and pathophysiological processes. In order to obtain direct evidence for NO functions in vivo, we have developed 4,5-diaminofluorescein (DAF-2) as a novel fluorescent indicator for NO. Green-fluorescent triazolofluorescein formed by the reaction of NO and DAF-2 affords high sensitivity for NO (detection limit: 5 nM). Membrane-permeable DAF-2 diacetate (DAF-2 DA) was loaded into activated rat aortic smooth muscle cells, where the ester bonds are hydrolyzed by intracellular esterase, generating DAF-2. The fluorescence in the cells increased in a NO concentration-dependent manner. This imaging method should be useful for studies of the dynamic biological actions of NO at the molecular level with fine temporal and spatial resolution.

**KEY WORDS** nitric oxide; imaging; fluorescent indicator; diaminofluorescein; vascular smooth muscle cell

Nitric oxide (NO) plays various roles as an intra- and intercellular signaling molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in the cardiovascular system and the central and peripheral nervous systems, as well as in immunological responses.[1–3] However, many proposed physiological roles of NO have not been proved directly by measuring NO. One of the reasons for this is the difficulty in direct, real-time detection of NO. Although several methods of detecting NO, which is unstable and produced at low concentration, have been developed,[4–7] a new method is required which is appropriate for studies in living cells in terms of selectivity, sensitivity, and experimental feasibility. The development of fluorescent indicators has played a major role in visualizing $Ca^{2+}$ in living cells.[8] Here, we report the design and synthesis of a fluorescent NO indicator based on the reactivity of NO. First, the reactivity of NO was examined in order to find a suitable reaction for selective NO trapping. We found that aromatic amines react with NO in the presence of dioxygen to produce the corresponding triazenes,[9] and the corresponding triazole ring compounds are generated from aromatic vicinal diamines under neutral conditions. We developed the new fluorescent indicator DAN-1 EE for bioimaging of NO[10] and found some problems, such as cytotoxicity and strong autofluorescence owing to the requirement for excitation with UV light, small extinction coefficient, and poor solubility in neutral buffer.

Fluorescein is widely used in biology as a fluorophore because of its convenient wavelengths for biological measurement, high extinction coefficient, and high fluorescence quantum yield in water. Fluoresceinamine was reported to show quenched fluorescence, but the conversion of amine to amide results in fluorescent properties.[11] This result implies that the fluorescence was quenched because of the electron-donating group attached to the phthalic ring of fluorescein. However, when the electron-donating group was transformed into a less electron-donating group, the fluorescence was recovered. Based on these findings, we designed and synthesized 4,5-diaminofluorescein (DAF-2)[12] as an indicator for NO. In other words, we considered that the electron donation of the functional groups

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attached to fluorescein would be reduced by reaction with NO via triazole ring formation, leading to an NO concentration-dependent enhancement of fluorescence.

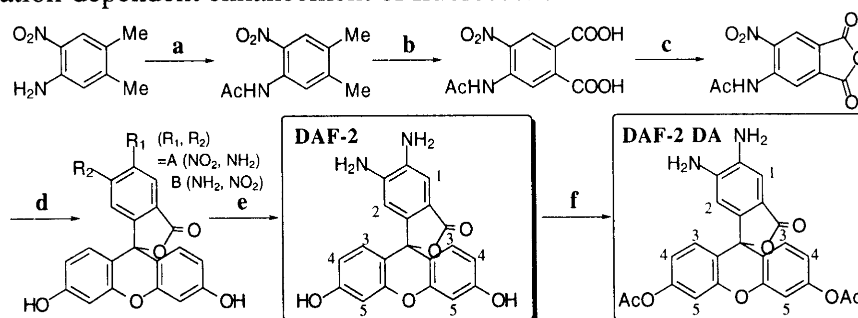


Chart a) $Ac_2O$, AcOH, 97% b) $KMnO_4$, $MgSO_4$, $H_2O$, 59% c) AcCl d) resorcinol; $ZnCl_2$; HCl, $H_2O$, A: 34%, B: 19% e) $Na_2S$, NaSH, $H_2O$, from A: 97%, from B: 30% f) $Ac_2O$, $Cs_2CO_3$, MeCN, 90%

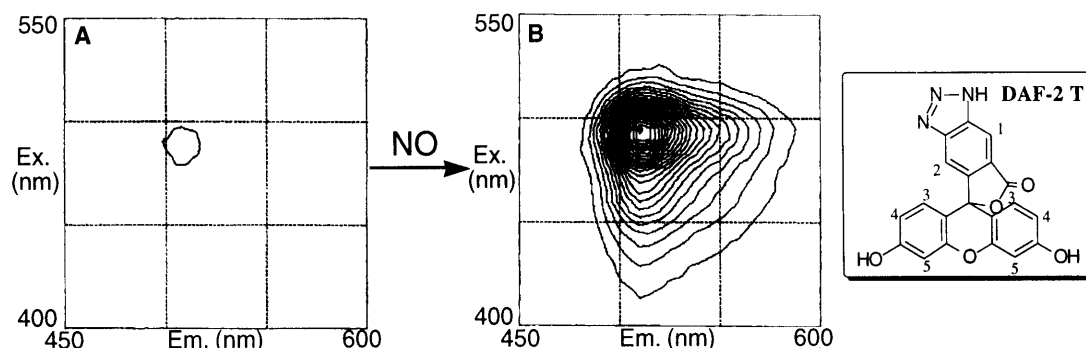


Fig. 1. Excitation and Emission Spectra of DAF-2 before and after the Reaction with NO

NO gas was bubbled into DAF-2 (1 μM) in sodium phosphate buffer (0.1 M, pH 7.4) for several seconds. The interval of contour lines is 10 units of fluorescence intensity.

The relatively nonfluorescent DAF-2 reacts rapidly with NO in oxygen-containing solution to yield its highly fluorescent product triazolofluorescein (DAF-2 T, Fig. 1).[13] Respectable extinction coefficients were obtained of $7.1 \times 10^4$ and $7.3 \times 10^4$ $M^{-1}$ $cm^{-1}$ at 486 and 491 nm, respectively, for DAF-2 and DAF-2 T. Quantum efficiencies of fluorescence of DAF-2 and DAF-2 T were also obtained of 0.005 and 0.92, respectively, by comparing the integral of the corrected emission spectrum of the test sample with that of a solution of fluorescein in 0.1 M NaOH, with the quantum efficiency assumed to be 0.85.[14] The increase in fluorescence intensity was dependent on the concentration of NO. The detection limit of NO by DAF-2 was 5 nM in neutral solution in the absence of biological samples as the result of examination of the calibration curve (data not shown). Stable oxidized forms of NO, such as $NO_2^-$ and $NO_3^-$, and other reactive oxygen species, such as $O_2^{\cdot -}$, $H_2O_2$, and $ONOO^-$, did not react with DAF-2 to yield any fluorescent product. Under physiological conditions DAF-2 T is not formed in the absence of NO.

We evaluated this new probe for real-time biological imaging of NO in activated rat aortic smooth muscle cells. In order to load the cells efficiently with the probe, it was necessary to prepare the diacetate (DAF-2 DA),[15] which can permeate readily into the cells,[16] where it is hydrolyzed by intracellular esterase to generate DAF-2.

The dye was applied to the imaging of NO in cultured rat aortic smooth muscle cells using a fluorescence microscope equipped with fluorescence filters for fluorescein chromophores. Figure 2 show that the fluorescence intensity in endotoxin- and cytokine-activated cells increased owing to DAF-2 T production from DAF-2 by reaction with NO. The increase was suppressed by the NO synthase inhibitor N-monomethyl-L-arginine (data not shown).

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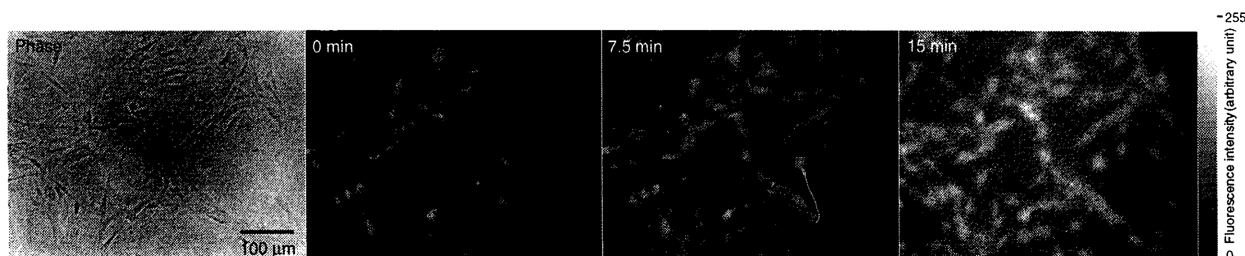


Fig. 2. Bright-field and Fluorescence Images of Activated Rat Aortic Smooth Muscle Cells Loaded with DAF-2 DA

For activation of the cells, a cytokine cocktail containing final concentrations of 25 U/ml interleukin-1β, 30 ng/ml tumor necrosis factor-α, 150 U/ml interferon-γ, and 12.5 μg/ml lipopolysaccharide was added to the medium. After 12-h incubation, the activated cells were used for the experiment. The cells were incubated for 1 h at 37°C in Krebs-Ringer phosphate buffer (KRP) containing 10 μM DAF-2 DA (0.2% DMSO was used as a cosolvent) for loading, washed with KRP, and placed in KRP containing L-Arg. They were mounted on an inverted fluorescence microscope equipped with an excitation filter (490 nm), a dichroic mirror (505 nm), and a long-pass emission filter (515 nm). Optical signals were recorded with an MCID (Imaging Research Inc., St. Catharines, Ontario, Canada), which is an imaging system including a charge-coupled device (CCD) camera.

Judging from the determination of NO evolved in the supernatant (data not shown), it was estimated that NO was produced at the rate of 2 fmol/h by a single cell under these conditions. It is highly likely that the concentration of NO in the cell before diffusion is considerably higher than that in the medium thus examined. Real-time visualization of the production and diffusion of NO with our new fluorescent indicator is useful for direct analysis of the cellular functions of NO.

## ACKNOWLEDGMENT

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12) Spectral data of DAF-2; $^1$H-NMR (DMSO-$d_6$, 300 MHz) δ: 5.00 (s, 2H, $NH_2$), 5.58 (s, 2H, $NH_2$), 6.07 (s, 1H, 2-H), 6.52 (dd, 2H, $J$ = 8.6, 2.2 Hz, 4-H), 6.60 (d, 2H, $J$ = 8.6 Hz, 3-H), 6.60 (d, 2H, $J$ = 2.2 Hz, 5-H), 6.89 (s, 1H, 1-H), 9.99 (s, 2H, OH). MS *m/z*: 362 ($M^+$). *Anal.* Calcd. for $C_{20}H_{14}N_2O_5 \cdot 0.6\ H_2O$: C, 64.37; H, 4.11; N, 7.51. Found: C, 64.12; H, 4.00; N, 7.55.
13) Spectral data of DAF-2 T; $^1$H-NMR (DMSO-$d_6$, 300 MHz) δ: 6.50 (dd, 2H, $J$ = 8.6, 2.4 Hz, 4-H), 6.59 (d, 2H, $J$ = 8.6 Hz, 3-H), 6.68 (d, 2H, $J$ = 2.4 Hz, 5-H), 7.68 (s, 1H, 2-H), 8.61 (s, 1H, 1-H), 10.09 (s, 2H, OH). MS *m/z*: 373 ($M^+$).
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