Spontaneous Increases in the Fluorescence of 4,5-Diaminofluorescein and Its Analogs: Their Impact on the Fluorometry of Nitric Oxide Production in Endothelial Cells

Nobuko Gan, Tsuyoshi Hondou, and Hidetake Miyata*

Department of Physics, Graduate School of Science, Tohoku University; Aramaki, Aoba-ku, Sendai 980–8578, Japan.

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We studied the spontaneous increase in the fluorescence intensity of 4,5-diaminofluorescein. A slow, steady increase in fluorescence continued for at least 125 h, and this increase was accompanied by ca. 2 nm red shift in the peak of emission spectrum. The spontaneous increase also occurred to diaminohodamine-4M and a fluorinated form of diamino fluorescein, which has been also used for the detection of nitric oxide (NO). We found that several factors (excitation light, pH etc.) did not alter the time course of this increase. Moreover, we found that this spontaneous increase can produce false-positive results when measuring low-rate nitric oxide production in human umbilical vein endothelial cells, and may confound the interpretation of results of NO production. We show that this adverse effect can be avoided by careful grouping of samples during measurement.

Key words nitric oxide; 4,5-diaminofluorescein; human umbilical cord endothelial cell

Nitric oxide (NO) production in cells, such as macrophages and endothelial cells was often measured using Griess reagent.1–5) Recently, a fluorescent indicator 4,5-diaminofluorescein (DAF-2) and its analogs have been introduced,4,5) and are now widely used.5–9) Upon nitrosation in the presence of dissolved oxygen DAF-2 is converted to the triazole form, DAF-2T (Fig. 1), and the quantum yield increases by >100-fold.4,5) Before the nitrosation, the fluorescence of DAF-2 arising from a fluorescein moiety is quenched due to electron donation from the diaminobenzene moiety, but nitrosation releases the quenching effect.4) The detection limit of NO with DAF-2 is 5 nM.4) Thus, DAF-2 is highly sensitive to the presence of NO.

The high sensitivity of DAF-2 is especially advantageous when it is used with endothelial cells because these cells produce NO at rates much lower than those of activated macrophages. The low rate of NO production in endothelial cells results in very small increases in the fluorescence intensity, because most DAF-2 remains un-reacted, producing background fluorescence. Thus, these small increases require careful, accurate measurement of the level of fluorescence intensity.11–13) In this situation, the stability of the fluorescence intensity of un-reacted DAF-2 is extremely important.

We are investigating the effect of magnetic fields on NO production in human umbilical vein endothelial cells (HUVECs)6) using DAF-2. During our study, we noticed a spontaneous increase in the fluorescence intensity of DAF-2 and its analogs in the absence of exogenous NO. Although the degree of the spontaneous increase is small compared with the large increase in the presence of sufficient amounts of NO (e.g., from NO releasing reagent), we realized that this spontaneous increase can lead to an erroneous conclusion if the amount of produced NO is small. Here, we report the results of phenomenological study of the spontaneous increase and evaluation of the effects of these increases on the measurement of NO production by HUVECs.

**MATERIALS AND METHODS**

**Fluorescent Reagents and Other Chemicals** DAF-2, diaminohodamine-4M (DAR-4M) and a fluorinated form of diamino fluorescein (DAF-FM), which are both previously developed analogs of DAF-2,4,5) were purchased from Sekisui Medical Co., Ltd. (Tokyo, Japan). DAF-2T was purchased from Enzo Biochem, Inc. (New York, NY, U.S.A.). Stock solutions of all fluorophores were prepared in dimethylsulfoxide (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and were stored at −80°C. All fluorescent materials were handled under reduced light. Concentrations of each fluorophore in the stock solutions were: 5 mM for DAF-2, 4.7 mM for DAR-4M, 6.9 mM for DAF-FM, and 0.5 mM for DAF-2T. Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. and were of analytical grade. Krebs–Ringer phosphate buffer (KRP) contained 120 mM NaCl, 4.8 mM KCl, 0.54 mM CaCl2·2H2O, 1.2 mM MgSO4·7H2O, 11 mM glucose, 15.9 mM Na2HPO4·12H2O (pH 7.2).4) Ca–Mg-free phosphate-buffered saline (pH 7.2) contained 136.9 mM NaCl, 2.68 mM KCl, 8.1 mM Na2HPO4·12H2O, and 1.47 mM KH2PO4. All buffers were prepared with highly purified water (MilliQ Simplicity UV, Nihon Millipore K.K., Tokyo, Japan).

![Fig. 1. Chemical Structure of DAF-2 and DAF-2T](image)

In DAF-2, the fluorescein moiety is the fluorophore; the diaminobenzene moiety reacts with N2O3, which is an oxidation product of NO, to become highly fluorescent DAF-2T with a triazole moiety. Before the nitrosation, the fluorescence is quenched by the electron donated from diaminobenzene moiety; nitrosation reduces the electron-donating ability, thereby enhancing the fluorescence intensity.
of DAF-2 were employed in a previous study.\textsuperscript{14}  

Similar optical conditions for the measurement of fluorescence spectra of buffer were acquired every 1 nm. Fluorescence spectra of buffer except for the case of DAR-4M. In the case of the use of DAR-4M, emission intensities were measured at 20°C at 575 nm with 515 nm as an emission wavelength, and emission wavelengths were set at 560 nm and 575 nm (spectral bandwidth=5 nm for both channels), respectively. Data were acquired every 2 s with continuous illumination of excitation light except for the case of DAR-4M. In the case of the use of DAR-4M, data were acquired every 10 s. To initiate the recording of a fluorescence signal, a cuvette containing appropriate buffer was placed in the cuvette holder. Approximately 1 min after the start of recording of the signal, a stock solution of DAF-2 (or DAF-2T, DAR-4M, or DAF-FM, depending on the measurement) was added (final concentration=1 $\mu$M).  

Measurement of Fluorescence Spectra  
Excitation spectra of DAF-2 and DAF-2T were acquired between 400 and 550 nm with 515 nm as an emission wavelength, and emission spectra were acquired between 450 and 600 nm with 495 nm as an excitation wavelength. In both cases, the bandwidth for both excitation and emission channels were 5 nm and signals were acquired every 1 nm. Fluorescence spectra of buffer alone were also measured under the same condition. Similar optical conditions for the measurement of fluorescence spectra of DAF-2 were employed in a previous study.\textsuperscript{14}  

Measurement of Time–Course of Fluorescence Change  
To obtain the time course of fluorescence of DAF-2, DAF-2T, or DAF-FM, excitation and emission wavelengths were set at 495 nm and 515 nm (spectral bandwidth=5 nm for both channels), respectively. For DAR-4M excitation and emission wavelengths were set at 560 nm and 575 nm (spectral bandwidth=5 nm for both channels), respectively. Data were acquired every 2 s with continuous illumination of excitation light. Approximately 1 min after the start of recording of the signal, a stock solution of DAF-2 (or DAF-2T, DAR-4M, or DAF-FM, depending on the measurement) was added (final concentration=1 $\mu$M).  

HPLC Analysis  
DAF-2 incubated at 17–18°C in darkness (hereafter called incubated DAF-2), non-incubated DAF-2, or DAF-2T was loaded onto the octadecylsilane column (InertSustain C18 column) and was eluted under an isocratic condition (10 mM sodium phosphate, pH 7.4–acetoniitrile=96:4).\textsuperscript{5}  

Reduction of Dissolved Oxygen in KRP Buffer To reduce dissolved oxygen in KRP, KRP was saturated with nitrogen (N$_2$) gas at room temperature (18–20°C): the oxygen concentration was reduced from 8.8 to 0.6 mg/L after the introduction of N$_2$ gas. Then, the N$_2$-saturated KRP was immediately transferred to a cuvette with a screw cap, DAF-2 (1 $\mu$M) was added, and the cuvette was placed in the spectrofluorophotometer for fluorescence measurement.

Fluorescence Analysis  
Excitation and emission spectra shown in Figs. 3A and B were obtained by taking the difference in the spectra in the presence and absence of fluorophore (i.e., buffer alone). The spectra were characterized by their shapes and peak wavelengths.

In the analysis of the time course of the fluorescence, the normalized rate of fluorescence increase was obtained as follows. First, we performed linear fitting of the time course of fluorescence change between 150 and 500 s. Then, the slope of the fitted line (the rate of fluorescence increase) was divided by the ordinate value, which is also obtained from the fitting procedure, and the resultant value is designated as the normalized rate. The normalized rates were used for comparison of the fluorescence increase obtained under different experimental conditions.  

Measurement of NO Production in HUVEC  
NO production by HUVECs (from Lonza Japan, Tokyo) plated in 6 cm cell culture dishes was cultivated in a CO$_2$ incubator (Model 5140, NAPCO, Chicago, IL, U.S.A.). Prior to the NO measurement, cell culture medium was removed from each dish, and cells were washed with KRP. Then, 3 mL KRP containing 1 $\mu$M DAR-4M was added to each cell culture dish. Thus, all dishes carried the same amount of KRP containing the same concentration of DAR-4M. After identification numbers (1 to 6) were assigned to these dishes, they were divided into two groups (hereafter designated Groups 1 and 2; 3 dishes in each group). The two groups of dishes were placed side-by-side in the same incubator. After 1 h of incubation KRP was collected from each dish into test tubes, and individual fluorescence intensities were measured at 20°C at 575 nm with an excitation wavelength of 560 nm (spectral bandwidth was 5 nm for both channels) according to the number assigned to the dishes (i.e., from 1 to 6). This was followed by a measurement in which the samples were measured in the reverse order (from 6 to 1).

RESULTS  

Spontaneous Increase in DAF-2 Fluorescence  
As shown in Fig. 2A, the fluorescence intensity of un-reacted DAF-2 gradually increased with time. Figure 2B shows that the steady increase continued for at least 125 h. As shown in Fig. 2C the fluorescence of DAR-4M and of DAF-FM also increased spontaneously.

Changes in Excitation and Emission Spectra  
We obtained the excitation and emission spectra immediately before the start of the measurement of the time course (hereafter called pre-excitation and pre-emission spectrum, respectively) and compared them with the spectra obtained at 125 h after the start of measurement of the first time point (post-excitation and post-emission spectrum). Figure 3A shows that the shapes of the pre- and post-excitation spectra of DAF-2 (gray line and black dots, respectively) were similar to each other, but the post-excitation spectrum of DAF-2 was shifted by ca. 1 nm toward a longer wavelength (Fig. 3B). The shapes of pre- and post-emission spectra of DAF-2 (gray line and black dots, respectively) were also similar to each other (Fig. 3A), but the post-emission spectrum shifted by ca. 2 nm toward a longer wavelength; the peak wavelength shifted from 509 to 511 nm (Fig. 3B). As shown in Table 1, the emission peak shifted gradually with time, suggesting some change occurred to DAF-2 throughout the incubation period.

Figure 3A also shows that the shape of excitation spectrum of DAF-2T (black line) was quite similar to the pre-excitation spectrum of DAF-2 (gray line), but as shown in Fig. 3B, its peak wavelength (493 nm) was red-shifted about 3 nm. The shape of emission spectrum of DAF-2T was also quite similar.
to that of the pre-emission spectrum of DAF-2, but the peak (514 nm) was red-shifted about 5 nm. Thus, the changes occurring in the fluorescence spectra of DAF-2 after the incubation in darkness were qualitatively similar to those occurring in DAF-2 after nitrosation, but the degree of changes was smaller.

**HPLC Analysis of DAF-2 after Incubation in KRP**

DAF-2 (5 µM) in KRP was kept in darkness for 120 h. The incubated DAF-2 exhibited the increase in the emission intensity and red-shift of the peak of the emission spectrum. When this incubated DAF-2 was examined by HPLC, one major peak appeared in the chromatogram, as shown in Fig. 4A. Figure 4B shows that the mixture of non-incubated DAF-2 and DAF-FM was measured at 515 nm with the excitation at 495 nm, and that of DAR-4M was measured at 575 nm with the excitation at 560 nm. The overshoots in the beginning of the time courses resulted from the electrical transience of the fluorescence spectrophotometer. During the intermittent measurement (panel B), samples were kept at 15°C in darkness between measurements.

Fig. 2. A Typical Example of the Time Course of Spontaneous Increase of Fluorescence Intensity of DAF-2 Measured over 40 min (A), the Increase Measured Intermittently over 125 h (B), the Increase Observed with DAF-2 Analogs, DAR-4M and DAF-FM (C)

Fluorescence intensity (in arbitrary units (a.u.)) of DAF-2 and DAF-FM was measured at 515 nm, with the excitation at 495 nm, and that of DAR-4M was measured at 575 nm with the excitation at 560 nm. The overshoots in the beginning of the time courses resulted from the electrical transience of the fluorescence spectrophotometer. During the intermittent measurement (panel B), samples were kept at 15°C in darkness between measurements.

Fig. 3. Normalized Excitation and Emission Spectra of DAF-2 before and after 125 h Incubation and Those of DAF-2T (A), and the Same Spectra Shown in an Expanded Scale (B)

The excitation spectra were measured with the emission wavelength of 515 nm; the emission spectra were measured with the excitation wavelength of 495 nm. For the sake of comparison of the peak positions, spectra were normalized to individual peak values. DAF-2 spectra before and after the 125 h incubation are represented by gray lines and black dotted lines, respectively, and those of DAF-2T are represented by black lines. In (B) part of each spectrum is shown in an expanded scale.

Table 1. Shift of Peak Wavelengths of Excitation and Emission Spectra of DAF-2

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Ex max (nm)</th>
<th>Em max (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>490</td>
<td>509</td>
</tr>
<tr>
<td>2</td>
<td>490</td>
<td>510</td>
</tr>
<tr>
<td>4</td>
<td>490</td>
<td>510</td>
</tr>
<tr>
<td>8</td>
<td>490</td>
<td>510</td>
</tr>
<tr>
<td>25</td>
<td>490</td>
<td>510</td>
</tr>
<tr>
<td>50</td>
<td>490</td>
<td>511</td>
</tr>
<tr>
<td>77</td>
<td>491</td>
<td>511</td>
</tr>
<tr>
<td>125</td>
<td>490</td>
<td>511</td>
</tr>
</tbody>
</table>

(Fig. 4C).

Effect of Several Factors on the Spontaneous Increase

The above experiments were done using KRP as the buffer. However, in Ca–Mg-free phosphate-buffered saline, the spontaneous increase in fluorescence intensity and similar peak shift in excitation and emission spectra also occurred (not shown). Therefore, Ca and Mg ions were unlikely to cause the spontaneous increase.

We assessed whether the excitation light promoted the spontaneous increase in fluorescence in two independent experiments. In one experiment, one sample was continuously illuminated at 495 nm for 33 min and another sample was kept in the dark over the same period. In both cases, the fluorescence
The intensity measured at 510 nm (excitation at 495 nm) increased by 6.6% over the intensity before the treatment. In the other experiment, we evaluated whether the normalized rate of fluorescence increase, as defined in ‘Fluorescence analysis’ in Materials and Methods, was affected by the change in the intensity of excitation light by inserting neutral density filters in the excitation light path. These filters attenuated the intensity of excitation light to 15% of the original intensity, and the normalized rate showed a slight decrease, as shown in Fig. 5. However, the linear regression line \( R^2 = 0.70 \) does not pass the origin. Therefore, we concluded that the spontaneous increase was unlikely to be promoted by the excitation light within the examined range of light intensity.

We assessed whether the pH of KRP changed over a 30-min time period. The pH decreased from 7.22 to 7.16 during the 30 min at 22°C. Around pH 7.2, to which our buffers were adjusted, DAF-2 fluorescence plateaued, and then it decreased with decreasing pH. However, the fluorescence intensity of DAF-2 increased despite the decrease in pH of the buffer. In addition, Fig. 1C shows that the fluorescence intensity of DAF-FM increased over the course of the 40-min measurement. The fluorescence intensity of DAF-FM is reportedly unaffected by changes in pH values over 6.0. Taken together, changes in pH were unlikely to cause the spontaneous increase in fluorescence.

In KRP saturated with \( N_2 \) gas, the dissolved oxygen concentration was reduced to ca. 1/15 of the un-treated KRP, but the fluorescence increase also occurred in deoxygenated KRP: the time course of fluorescence increase was indistinguishable from that obtained with un-treated KRP (Fig. 6). Hence, the possibility is remote that the dissolved oxygen caused or promoted the spontaneous increase in fluorescence.

### The Impact of Spontaneous Increases on Fluorometry of NO Production in HUVECs

We initially noticed the spontaneous increases in fluorescence intensity when evaluating the effect of magnetic fields on NO production in HUVECs. As described in Materials and Methods, samples were KRP solutions containing DAR-4M that was taken from 6 cell culture dishes kept in the same incubator without magnetic
This was because DAR-4M increased (Fig. 2C). To minimize the effect of the increase on the measurement, intensity was not linear over time. Table 2 ("bias to increase with dish number, as shown in Fig. 7B. This was to be higher than those of samples measured earlier (Fig. 7A).

Believe that this false-positive result arose from the biased division of the cell culture dishes: the dishes in Group 1 had smaller numbers (1–3) than those (4–6) in Group 2, and were hence measured earlier. We performed eight similar experiments, including the experiment shown in Table 2, and in five cases obtained statistically significant differences (p=0.01) in the averaged fluorescence intensity between two replicate groups of samples.

In another experiment (Table 2, “less biased grouping of dishes”), the dishes were carefully divided into two groups to reduce the number bias ((2–4) vs. (1, 5, 6) in this particular case), and statistically significant differences were not observed (p=0.43). We carried out five experiments (including the one shown in Table 2) with this less biased grouping, and in only one case was the difference between two groups significant (p<0.01).

DISCUSSION

We found a spontaneous increase in fluorescence intensity of DAF-2 and its analogs, DAR-4M and DAF-FM. The fluorescence increase was accompanied by changes in the emission and excitation spectra of DAF-2. Several factors (Ca and Mg ions, excitation light, pH change of solution and dissolved oxygen) were found not to affect the time course of the fluorescence increase. HPLC analysis showed that DAF-2 incubated in darkness was chromatographically indistinguishable from non-incubated DAF-2. We finally showed that the spontaneous increase of DAF-2 fluorescence can introduce false-positive results into measurements of low rate NO production in HUVEC, but this can be avoided by proper handling.

It has been demonstrated that the fluorescence intensity of DAF-FM spontaneously increases in the absence of NO and that the accompanying change in fluorescence spectra is not different from that of nitrosated DAF-2. Exposure of the fluorophore to a dim light (200 lx) for 150 min promoted the fluorescence increase by ca. 100%. The spontaneous increase has thus been attributed to the auto-oxidation of DAF-FM, which is promoted by light.

We showed that the fluorescence increase was little affected under the oxygen-depleted condition, suggesting that the auto-oxidation mechanism did not work in the fluorescence increase in the present case. We also showed that the fluorescence increase was little affected by the excitation light. In our study, however, the intensity of excitation light was estimated to be ca. 10^4 lx, and therefore, the possibility remains that the intensity exceeded the range within which the promoting effect of light existed. We could not test this possibility by sufficiently reducing the intensity of excitation light, because the fluorescence signal would be too low to be reliably detected even with the highest sensitivity in the fluorescence spectrophotometer. However, as described in Results, DAF-2 continuously exposed to, and not exposed to,

![Image](56x492 to 281x744)

**Table 2. A Typical Example of the Effect of Spontaneous Increases in Fluorescence Intensities on Measurement of NO in Identical Samples**

<table>
<thead>
<tr>
<th>Biased grouping of dishes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Less biased grouping of dishes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p Value</th>
<th>Biased grouping of dishes&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Less biased grouping of dishes&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>Group 2</td>
<td></td>
<td>Group 1</td>
<td>Group 2</td>
<td></td>
</tr>
<tr>
<td>Averaged intensity</td>
<td>33.6±0.05</td>
<td>35.6±0.27</td>
<td>0.01</td>
<td>33.7±2.3</td>
<td>34.5±0.12</td>
</tr>
<tr>
<td>Dish number&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(1, 2, 3)</td>
<td>(4, 5, 6)</td>
<td></td>
<td>(2, 3, 4)</td>
<td>(1, 5, 6)</td>
</tr>
</tbody>
</table>

<sup>a</sup> These experiments were carried out separately. <sup>b</sup> Numbers in parenthesis indicate the numbers assigned to each dish in the group.
the excitation light exhibited the same degree of enhancement of fluorescence. Hence, we conclude that the excitation light is not the primary cause for the spontaneous increase. The two fluorine atoms bound to the fluorescein moiety in DAF-FM<sup>5</sup> may be the reason for the difference between the previous and present results.

It has been reported that Ca or Mg ions greatly enhance DAF-2 fluorescence. This phenomenon has been attributed to the acceleration of the release of NO from NO releasing reagents by divalent cations. In our case, no exogenous NO source was present. The atmospheric NO concentration (<0.1 ppm) will give an NO concentration in the aqueous phase of ca. 0.2 nm or less, which is far below the detection limit (5 nm)<sup>3</sup> of NO with DAF-2.

One would expect on the basis of the spectral shift that a small amount of DAF-2 was converted to DAF-2T after the incubation in darkness. However, the incubated DAF-2 could not be chromatographically distinguished from the non-incubated DAF-2 under the condition where DAF-2 was well separated from DAF-2T. Thus, the nature of chemical change seemed to be different from that which accompanies the nitrosation.

The mechanism of fluorescence change remains to be clarified. DAF-2 has been shown to react with reducing agents such as dehydroascorbic acid and ascorbic acid, forming a compound with a fluorescence emission spectrum that is indistinguishable from that of DAF-2 after nitrosation. It has been proposed that the binding of dehydroascorbic acid to the dianisobenzene moiety causes the spectral change. In the present study, the change in fluorescence properties of DAF-2 also implies some chemical change. But the subtleness of the spectral change and the unaltered HPLC chromatogram suggest that the presumed chemical change seems to be less prominent than the changes accompanying the transformation of DAF-2 to DAF-2T or the formation of a dehydroascorbic acid adduct.

The spontaneous fluorescence increase of DAF-2 and its analogs can affect the measurements of NO in the cell with a low rate of NO production. The use of DAF-2 with such cells has been cautioned because the background fluorescence of DAF-2 can interfere with the estimation of NO concentration. We showed that the spontaneous increase introduces a systematic error in measurement with such cells, but the error can be minimized by introducing a proper randomization, as illustrated. Thus, we believe that with these cautions, DAF-2 is still a highly useful reagent in the measurements of nitric oxide.

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