**Analytical Biochemistry**

**Detection system of the intracellular nitric oxide in yeast by HPLC with a fluorescence detector**

---Manuscript Draft---

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<td>Corresponding Author:</td>
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<td>Hiroshi Takagi</td>
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**Abstract:**

Nitric oxide (NO) is an important signaling molecule involved in various biological phenomena in many organisms. The physiological functions and metabolism of NO in yeast, a unicellular microorganism, are still unknown, mainly because it is difficult to analyze the intracellular NO levels accurately. Here, we developed a new method of more accurately measuring NO content in yeast cells by treating the cells with an NO-specific fluorescence probe followed by high-performance liquid chromatography with fluorescence detection (HPLC/FLD). This approach successfully detected and quantified the NO content inside yeast cells treated with an NO donor. Moreover, the HPLC/FLD analysis indicates that the fluorescence induced under some environmental stress conditions, such as ethanol, vanillin, and heat-shock, was not derived from NO. The HPLC/FLD method developed in this study provides a new strategy for measuring the intracellular NO concentration with higher accuracy.

**Suggested Reviewers:**

- Hideshi Ihara  
  ihara@b.s.osakafu-u.ac.jp  
  He specializes the detection method of intracellular NO.

- Yasuteru Urano  
  uranokun@m.u-tokyo.ac.jp  
  He is an expert for development of fluorescent probes for reactive molecular species including nitric oxide.

- dennis stuehr  
  stuehrd@ccf.org  
  He is deeply involved in biochemistry for nitric oxide.

**Response to Reviewers:**
March 25, 2020

Dear Dr. Steven Smith (Executive editor),

Thank you very much for reviewing our manuscript entitled "Detection system of the intracellular nitric oxide in yeast by HPLC with a fluorescence detector". We certainly accept your valuable comments and have modified the manuscript accordingly, which included the rectified figure and additional table and statements. Our responses to all the comments from both you and two Referees are also described, item by item, in the attached sheet.

We greatly appreciate both your help and that of the reviewers concerning improvements to this paper. We believe that the revised manuscript meets with your approval for publication in Analytical Biochemistry.

Sincerely,

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March 25, 2020

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Editor and Reviewer comments:

Reviewer #1:
The authors reported a detection system for intracellular nitric oxide in yeast by HPLC with a fluorescence detector. Major revision is necessary before the publication of this manuscript due to many drawbacks. We are so grateful to you for giving us valuable comments concerning our manuscript. According to your helpful suggestions, we have modified the manuscript appropriately.

1. In the Abstract, provide the detection range of NO concentration and the limit of detection based on the use of this proposed detection system. We have added the detection limit (6 nM) to the text (L31-32 and L175-176).

2. In the Introduction, elaborate the novelty and research motivation of this manuscript clearly, focusing on the detection methods of NO in biological samples and intracellular NO. We have revised the manuscript accordingly (L64-65, L67-72, L76 and L87-95).

3. In the captions of all figures, “Fluorescence” can be abbreviated as “FL” simply. We have changed “Fluorescence intensity” to “FL intensity” in Figures 1, 2, and 4.

4. Provide a Table to summarize and compare the performances for NO detection, based on previous NO detection methods and this proposed detection system. We have summarized the sensitivity, advantages, and disadvantages of the previous methods and proposed system here in new Table 1 (L64-65).


6. The authors should revise the manuscript to avoid language errors, especially grammar and spelling errors. The revised manuscript needs to carefully check and review by some experts and experienced authors. We have revised the manuscript carefully.
Reviewer #2:
The authors developed a new method for quantified measurement of intracellular NO release by HPLC/FLD. This study is rather complete and well performed. The structure of the fluorescent probe should be provided in a Figure.

We are very pleased to note your positive comments to our manuscript. Following your suggestion, we have added the structures of DAF-FM DA, DAF-FM, and DAF-FM-T in Figure 1A.
Highlights

- We developed an NO measurement system using HPLC and a fluorescence NO probe.
- Our measurement system successfully quantified the intracellular NO level in yeast.
- The previous method using flowcytometry produced false positives for NO detection.
Environmental stresses

Exogenous NO

DAF-FM DA

DAF-FM

NO

Fluorescent

DAF-FM-T

Extraction

HPLC/FLD

No false positive
Detection system of the intracellular nitric oxide in yeast by HPLC with a fluorescence detector

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Short title
Detection system of the intracellular nitric oxide in yeast
Abstract

Nitric oxide (NO) is an important signaling molecule involved in various biological phenomena in many organisms. The physiological functions and metabolism of NO in yeast, a unicellular microorganism, are still unknown, mainly because it is difficult to analyze the intracellular NO levels accurately. Here, we developed a new method of more accurately measuring NO content in yeast cells with the detection limit of 6 nM, by treating the cells with an NO-specific fluorescence probe followed by high-performance liquid chromatography with fluorescence detection (HPLC/FLD). This approach successfully detected and quantified the NO content inside yeast cells treated with an NO donor. Moreover, the HPLC/FLD analysis indicates that the fluorescence induced under some environmental stress conditions, such as ethanol, vanillin, and heat-shock, was not derived from NO. The HPLC/FLD method developed in this study provides a new strategy for measuring the intracellular NO concentration with higher accuracy.

Keywords

Nitric oxide; Fluorescence probe; Methodology; Yeast; Environmental stress

Abbreviations

DAF-FM, 4-amino-5-methylamino-2’,7’-difluorofluorescein; DAF-FM DA, 4-amino-5-methylamino-2’,7’-difluorofluorescein diacetate; DAF-FM-T, 4-amino-5-methylamino-2’,7’-difluorofluorescein triazole; NO, nitric oxide; HPLC, high-performance liquid chromatography; FLD, fluorescence detector; FCM, flowcytometry.
1. Introduction

Nitric oxide (NO) is a ubiquitous signaling molecule involved in a variety of biological events in many kinds of organisms [1,2]. NO is synthesized from L-arginine as a substrate by NO synthase (NOS) in mammalian cells [3,4]. Some bacteria possess NOS orthologues to produce NO [5,6]. Although most plants do not possess NOS orthologues on their genomes, they can reduce nitrite/nitrate to NO by nitrite reductase and nitrate reductase, respectively [7,8]. The budding yeast *Saccharomyces cerevisiae*, which is an important microorganism as a model for higher eukaryotes and pathogenic fungi, has also been reported to produce NO, even though, like plants, it lacks the NOS orthologue on its genome [9–12]. Previous studies found that NO promotes cell death under an H$_2$O$_2$ treatment condition in yeast [9,11,12]. On the other hand, we have shown that NO confers tolerance to high-temperature stress on yeast cells [10,13].

However, the physiological functions and metabolism of NO in yeast are still under investigation. One of the reasons for the slow progress of NO research in yeast is the difficulty of measuring intracellular NO content accurately. Previous studies have thus adopted various strategies for NO-content estimation in yeast (Table 1). For example, because NO is oxidized to nitrite and nitrate under aerobic conditions, nitrite and nitrate have been measured as indices of NO generation, either colorimetrically (the Griess assay) or fluorometrically (using 2,3-diaminonaphthalene) [9,10]. However, the nitrite/nitrate assay is interfered with environmental nitrite/nitrate contamination and its sensitivity is not high. Another approach to measure the NO concentration in yeast cells is to use NO-specific electrodes, which are widely used to estimate NO concentrations in mammalian tissues. In this method, however, the NO concentration must be measured extracellularly in a yeast cell suspension [9]. One of the most convenient methods for NO measurement in yeast is the use of the NO-specific probe 4-amino-5-methylamino-2′,7′-
difluorofluorescein (DAF-FM) and/or its diacetylated derivative DAF-FM DA, which is a cell-permeable form of DAF-FM (Fig. 1A) [14]. DAF-FM DA is hydrolyzed to DAF-FM by intracellular esterases after incorporation into the cells. DAF-FM is used in combination with fluorometric monitoring to measure the NO concentration in a solution or in an in vitro sample [15]. On the other hand, DAF-FM DA has been used to stain living cells to estimate the intracellular NO content, combined with evaluation by flow cytometry (FCM) and/or fluorescence microscopy [9–12]. Measurement methods using DAF-FM and/or DAF-FM DA have the advantages of high sensitivity and a convenient protocol. However, the accuracy of these methods is completely dependent on the reaction specificity of DAF-FM, even though no information has been published about the specificity of DAF-FM, particularly within cells. In fact, it has been reported that DAF-FM reacts with peroxynitrite, which is a reaction product of NO and superoxide anion [15]. As described above, the previous strategies adopted to measure the intracellular NO level in yeast is not sufficient in terms of sensitivity and specificity, although it is necessary to quantify NO in yeast cells with high sensitivity and accuracy for further progresses of NO research in yeast. Recently, a new fluorescence NO probe was developed and used to quantify the NO content in mammalian cells [16–18]. However, this is not commercially available yet and has never been used for yeast research. Therefore, we focused on the strategy which has been already used to quantify NO in yeast cells and modified it to develop the new NO measurement system with higher sensitivity and more strict specificity.

Here, we developed a new method to measure the intracellular NO content using high-performance liquid chromatography with a fluorescence detector (HPLC/FLD) combined with DAF-FM DA staining. Our HPLC/FLD analysis quantified the intracellular NO content in yeast when treated with an NO donor and indicated that the
previously reported method using FCM produced false positives under some conditions.

2. Materials and methods

2.1. Strains and media

The yeast *Saccharomyces cerevisiae* X2180-1A strain was used in this study [19]. Yeast cells were cultured in a synthetic minimal SD medium consisting of 2% glucose, 0.5% ammonium sulfate, and 0.17% BD Difco™ Yeast Nitrogen Base Without Amino Acids Ammonium Sulfate (Fisher Scientific).

2.2. Flowcytometry

The yeast strain was cultured in SD medium at 25°C until its exponential growth phase and then treated with 5 μM DAF-FM DA (Goryo Chemical) for 30 min at room temperature, followed by exposure to various stresses. For example, yeast cells were treated with 1 mM of an NO donor MAHMA NONOate and then incubated for 30 min. In the case of environmental stress conditions, yeast cells were collected and either resuspended in SD medium with 15% ethanol, 20 mM vanillin, or in SD medium with pre-heated at 50°C followed by additional incubation for 2 h, 2 h, or 10 min, respectively. After stress treatment, the cell suspension was subjected to FCM using BD Accuri C6 flow cytometer (Becton, Dickinson Bioscience).

2.3. NO measurement by HPLC/FLD

HPLC was performed using a LaChrom Elite system (Hitachi) with a fluorescence detector 5440 (Hitachi) and a LaChrom II C18 ODS column (4.6 mm × 150 mm) (Hitachi). The following gradient system was used for the detection and quantification of DAF-FM-T with a flow rate of 1 mL/min, using 20 mM potassium
phosphate buffer (pH 7.0) and acetonitrile as solvents A and B, respectively (time, % of solvent A): 0 min, 95%; 50 min, 80%; 50.1 min, 50%; 55 min, 50%; 60 min, 95%; 65 min, 95% (Method A). The same gradient system was used to examine the previously reported method [20] using 0.05% trifluoroacetic acid (TFA) aqueous solution and acetonitrile containing 0.05% TFA as solvents A and B, respectively (Method B). Fluorescence detection was performed with an excitation and emission wavelengths of 490 nm and 517 nm, respectively. All methods of HPLC were carried out at 25°C.

2.4. Preparation of the standard DAF-FM-T.

First, 50 μM of DAF-FM (Goryo Chemical) was mixed with 400 μM of an NO donor MAHMA NONOate (Cayman Chemical) [21] in 10 mM of (NH₄)HCO₃ (pH 7.9), and then incubated for 60 min at 37°C. After the reaction, 200 μL of the reaction solution was mixed with 800 μL of methanol, followed by the centrifugation at 20,400 × g for 20 min. The supernatant was filtered and subjected to HPLC/FLD. In order to draw the standard curve of DAF-FM-T, the serial dilutions of the resultant reaction solution prepared as above were used; these contained 50 μM of DAF-FM-T theoretically because of the short half-life time of MAHMA NONOate in a neutral buffer (1 min at pH 7.4 and 37°C) [21] were used.

2.5. Preparation of the yeast lysate for analysis with HPLC/FLD.

Yeast cells were cultured in SD medium containing 500 μM l-arginine at 30°C until the exponential growth phase. After washing with 0.1 M sodium phosphate buffer (NPB) (pH 7.0), cells with OD of 50 were treated with 50 μM DAF-FM DA (Goryo Chemical) in 0.1 M NPB (pH 7.0) for 30 min at room temperature. The DAF-FM DA-treated cells were diluted to OD of 1 in 0.1 M NPB (pH 7.0), followed by further
incubation with 1 mM MAHMA NONOate at 30°C for 30 min, or either 10% ethanol or
20 mM vanillin at 30°C for 2 h. For heat-shock treatment, the cells were incubated at 50°C
for 10 min after incubation with DAF-FM DA. After the stress treatment, the cells were
harvested, washed with, and suspended in 0.1 M NPB (pH 7.0), and then disrupted with
glass beads using Multi-Beads Shocker (Yasui Kikai). The supernatant after
centrifugation at 20,400 × g for 20 min was used for further analyses. Two volumes of
supernatant, whose protein concentrations were unified with 0.1 M NPB (pH 7.0), were
mixed with 8 volumes of methanol and vortexed, followed by the centrifugation at 20,400
× g for 20 min to remove proteins. The supernatant was filtered and subjected to
HPLC/FLD.

3. Results and discussion

3.1. Development of HPLC/FLD-based DAF-FM-T analysis system

Many previous studies have used a combination of DAF-FM DA treatment and
FCM and/or fluorescence microscopy to estimate intracellular NO levels in yeast and
other organisms [22–25]. However, these analyses could measure not only the
fluorescence from DAF-FM-T, which is the reaction product of NO and DAF-FM, but
also that derived from false positives. Therefore, we tried to use HPLC/FLD to develop
an analytic system that exclusively measures the fluorescence from DAF-FM-T (Fig. 1A).

First, we analyzed a reaction mixture of DAF-FM with the NO donor MAHMA
NONOate. Using the HPLC/FLD method, a clear fluorescence peak was detected at the
retention time of 19 min in the sample reacted with MAHMA NONOate, but no peak was
observed in the sample without MAHMA NONOate, indicating that this peak was DAF-
FM-T (Fig. 1B). We also drew a standard curve of DAF-FM-T in the concentration range
of 31 - 1,000 nM (Fig. 1C). Analysis of the curve demonstrated that the HPLC/FLD-based
NO quantification system was successfully developed. **Judging from the S/N ratio of peak area, the detection limit of DAF-FM-T was 6 nM.** Importantly, a gradient system using the same buffers as in the previous report (Method B) [20] did not show any fluorescence peaks even in the sample treated with MAHMA NONOate (Fig. 1B). This was likely because the fluorescence intensity from DAF-FM-T is highly dependent on the pH of samples, and thus DAF-FM-T has no or very weak fluorescence under acidic conditions [14]. Our HPLC/FLD system using a neutral buffer is compatible with the fluorescence detection of DAF-FM-T, and it therefore has much higher sensitivity than the previously reported systems.

3.2. **DAF-FM-T measurement generated in vivo**

We accurately measured the intracellular NO level in yeast treated with the NO donor MAHMA NONOate by measuring DAF-FM-T produced *in vivo* with our HPLC/FLD method. When yeast cells treated with DAF-FM DA were analyzed by FCM as a positive control, an increase in intracellular fluorescence was evident after exposure to an NO donor (Fig. 2A). Subsequently, we evaluated the DAF-FM-T content in yeast cells treated with an NO donor by the HPLC/FLD method developed in this study (Fig. 2B). We observed a marked fluorescence peak with the same retention time as the standard DAF-FM-T in the MAHMA NONOate-treated sample, and there was no peak in the untreated sample. These results indicate that the fluorescence peak observed in the NO donor-treated sample represented the same compound as DAF-FM-T, which was quantified as 5.0 nmol/mg protein. This is the first report of the successful detection and quantification of DAF-FM-T in yeast cells. In combination with mass spectrometry, the detection of the endogenously produced DAF-FM-T in further analyses enables us to identify the origin of NO within cells.
3.3. *DAF-FM-T* measurement under various stress conditions by FCM and HPLC/FLD

Next, we attempted to identify new environmental stress conditions that induce NO generation in yeast cells. Our FCM analyses using yeast cells treated with DAF-FM DA showed that the fluorescence intensity was dramatically enhanced after exposure to three stress conditions: ethanol, vanillin, or heat-shock (50°C) treatment (Fig. 3). These results suggest that NO is generated in yeast cells in response to these stimuli.

To confirm NO synthesis in response to these stresses, we examined the intracellular NO content using our HPLC/FLD method (Fig. 4). Unexpectedly, even though clear fluorescence peaks were observed in these samples, the retention times of these peaks were completely different from that of the standard DAF-FM-T. These results indicate that the fluorescence from the samples prepared under these stress conditions was not derived from NO. Although we have not yet identified the fluorescent compounds detected under these conditions, it is at least certain that the fluorescence peaks were dependent on DAF-FM DA, since no fluorescence peaks were detected in the absence of DAF-FM DA staining (data not shown). Therefore, these fluorescent compounds may have been derived from the reactions of DAF-FM with unknown reactive species in yeast cells. On the other hand, it is possible that these compounds were merely the product of an intracellular enzymatic reaction involving DAF-FM as a substrate, which was activated under these stress conditions. Thus, it will be necessary to identify these compounds in order to confirm this hypothesis. Such identification could also contribute to the investigation of unknown reactive species and/or to the development of new NO-specific fluorescence probes with higher specificity and accuracy.

4. Conclusions
We developed a new quantification method to measure intracellular NO levels by HPLC/FLD. Our HPLC/FLD system successfully detected and quantified the NO content in yeast cells treated with an NO donor. On the other hand, our HPLC/FLD method showed that the fluorescence signals derived from DAF-FM DA-stained cells observed in FCM were shown to be false positives under some conditions. The HPLC/FLD system described here provides a new strategy for analyzing the intracellular NO concentrations in yeast and other organisms, and has greater reliability than the previous methods using fluorescence probes of NO.

Declaration of competing interest
The authors declare no conflict of interests.

Acknowledgments
This work was supported by grants for a Grant-in-Aid for Young Scientists (B) (15K21165), Grant-in-Aid for Young Scientist (19K16129) from Japan Society for the Promotion of Science (JSPS) to R.N., and also a Grant-in-Aid for Scientific Research (S) (19H05639) and (A) (16H02601), Grant-in-Aid for Challenging Exploratory Research (19K22282) from Japan Society for the Promotion of Science (JSPS), and a Grant-in-Aid for Scientific Research on Innovative Area (Oxygen Biology) (26111009) from the Ministry of Education, Sciences, Sports and Technology (MEXT) to H.T.

References


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https://doi.org/10.1371/journal.pone.0113788.


R. I. Astuti, D. Watanabe, H. Takagi, Nitric oxide signaling and its role in


Figure legends

Fig. 1. New NO measurement system using HPLC/FLD. (A) Scheme of HPLC/FLD for the intracellular NO measurement in yeast. Yeast cells treated with DAF-FM DA followed by stress exposure were lysed and the extract was subjected to HPLC/FLD. (B) Chromatogram of the standard DAF-FM-T. The letter A or B shown in the chromatogram indicates the analysis with Method A or B, respectively. The black arrowhead indicates the peak of DAF-FM-T. The reaction solution of DAF-FM with MAHMA NONOate was analyzed by HPLC/FLD. The representative chromatogram from at least three independent experiments. (C) The standard curve of DAF-FM-T for NO quantification.

Fig. 2. NO measurement from the NO donor-treated yeast cells. (A) The result from FCM after exposure to an NO donor MAHMA NONOate. The representative result from two independent results was shown. (B) Chromatogram of the lysate from the NO donor-treated or untreated yeast cells, and that of the standard DAF-FM-T. The black arrowhead indicates the fluorescence peak of DAF-FM-T. The representative result from three independent results was shown.

Fig. 3. Intracellular fluorescence level observed with FCM using DAF-FM DA. Yeast cells treated with DAF-FM DA were exposed to (A) 15% ethanol for 2 h, (B) 20 mM vanillin for 2 h, or (C) 50°C for 10 min and then subjected to FCM. The representative histograms from at least two independent experiments were shown.

Fig. 4. Intracellular NO level analyzed by HPLC/FLD. Yeast cells treated with DAF-FM DA were exposed to (A) 10% ethanol for 2 h, (B) 20 mM vanillin for 2 h, or (C) 50°C
for 10 min, and then the extracts from the cells were analyzed by HPLC/FLD. The representative results from at least two independent experiments were shown.
Table 1. Summary of NO detection methods previously used for yeast research.

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<th>Sensitivity</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<td>NO electrode</td>
<td>0.06-5,500 nM [26]</td>
<td>Direct, continuous, sensitive</td>
<td>Uncertain specificity, size</td>
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<tr>
<td>Fluorometry</td>
<td>3 nM [14]</td>
<td>Fast, sensitive</td>
<td>Uncertain specificity</td>
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<td>Griess assay</td>
<td>500 nM</td>
<td>Cheap, fast</td>
<td>Indirect, low sensitivity</td>
</tr>
<tr>
<td>HPLC/FLD*</td>
<td>6 nM</td>
<td>Fast, sensitive, specific</td>
<td>Longer time than fluorometry</td>
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*A method developed in this study.*
Figure 1. Environmental Stresses lead to the production of NO, which induces DAF-FM-DA to convert into DAF-FM. This process is then extracted for HPLC/FLD analysis. The retention time (min) and FL intensity (A.U.) are used to measure the concentration of DAF-FM-T (nM), with a correlation coefficient of $R^2 = 0.9997$.
Fig. 2.
Fig. 3.
Fig. 4.
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Author Contributions

R.N. and H.T. conceived the study and designed the experiments. S.S., Y.Y., N.Y., Y.S. and K.K. performed the experiments. R.N. and H.T. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.
Declaration of competing interest

The authors declare no conflict of interests.