Visualization of NO$_3^-$/NO$_2^-$ Dynamics in Living Cells by Fluorescence Resonance Energy Transfer (FRET) Imaging Employing a Rhizobial Two-component Regulatory System*§

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Nitrate (NO$_3^-$) and nitrite (NO$_2^-$) are the physiological sources of nitric oxide (NO), a key biological messenger molecule. NO$_3^-$/NO$_2^-$ exerts a beneficial impact on NO homeostasis and its related cardiovascular functions. To visualize the physiological dynamics of NO$_3^-$/NO$_2^-$ for assessing the precise roles of these anions, we developed a genetically encoded intermolecular fluorescence resonance energy transfer (FRET)-based indicator, named sNOOOpy (sensor for NO$_3^-$ resonance energy transfer (FRET)-based indicator), employing NO$_3^-$/NO$_2^-$-induced dissociation of NasST involved in the denitrification system of rhizobia. The in vitro use of sNOOOpy shows high specificity for NO$_3^-$ and NO$_2^-$, and its FRET signal is changed in response to NO$_3^-$/NO$_2^-$ in the micromolar range. Furthermore, both an increase and decrease in cellular NO$_3^-$ concentration can be detected. sNOOOpy is very simple and potentially applicable to a wide variety of living cells and is expected to provide insights into NO$_3^-$/NO$_2^-$ dynamics in various organisms, including plants and animals.

Nitrate (NO$_3^-$) and nitrite (NO$_2^-$) are metabolites in the biological nitrogen cycle. In bacteria and plants, NO$_3^-$ is used as a substrate for respiration and/or assimilation. In animals, including humans, NO$_3^-$ and NO$_2^-$ (NO$_3^-$/NO$_2^-$) were recognized for a long time as being merely inert oxidants of nitric oxide (NO) (1). NO is a key signaling molecule that regulates a vast range of physiological functions, such as vascular homeostasis, neurotransmission, and host defense (2). Intriguingly, many studies over the last decade revealed that these inert NO$_3^-$/NO$_2^-$ species are physiologically recycled to form NO and other reactive nitrogen species through the "nitrate–nitrite–nitric oxide (NO$_3^-$–NO$_2^-$–NO) pathway" (3–5). Currently, NO$_3^-$/NO$_2^-$ are considered as stable reservoirs for NO-like bioactivity, and several beneficial aspects of NO$_3^-$/NO$_2^-$ in the treatment and prevention of cardiovascular diseases by restoring NO homeostasis are reported (5, 6).

As the regulation of NO$_3^-$/NO$_2^-$ in physiological processes is an attractive therapeutic target, it is important to understand NO$_3^-$/NO$_2^-$ in biological processes, how intracellular levels are regulated, and how they control cellular processes. The most frequently used method for NO$_3^-$/NO$_2^-$ measurement is based on the Griess reaction (7). The Griess method is also used to assess NO synthesis because of the immediate conversion of NO into NO$_3^-$/NO$_2^-$ (half-life 2–6 s) (8). Although measurement of NO$_3^-$/NO$_2^-$ by the Griess assay is simple and convenient, it is difficult to apply this method for in situ measurement in living cells because this method is generally used as the end point assay that involves several chemical reactions. As for mammalian cells, although the NO$_3^-$ influx into HeLa-derived cells at low pH conditions was observed by the patch clamp method (9), detections of the dynamics of NO$_3^-$/NO$_2^-$ in physiological processes are quite difficult by presently available methods.

In some microorganisms, nasST genes are clustered together with other genes involved in NO$_3^-$ assimilation (10–13). NasS and NasT are annotated as a NO$_3^-$/NO$_2^-$-responsive two-component system, where NasS is a NO$_3^-$/NO$_2^-$ sensor, and NasT is a transcription antiterminator. We have previously demonstrated that the NasS and NasT from the root nodule bacterium Bradyrhizobium japonicum form a stable complex (NasST) in the absence of NO$_3^-$/NO$_2^-$, and the formation of the NasS with NO$_3^-$ or NO$_2^-$ complex triggers release of the positive RNA-binding regulator NasT (13), which enhances the translation of proteins involved in NO$_3^-$ assimilation (Fig. 1A) (11). Herein, we report genetically encoded FRET-based NO$_3^-$/NO$_2^-$ biosensors that employ NasS and NasT (Fig. 1B). Using this system, we succeeded in monitoring the dynamics of NO$_3^-$/NO$_2^-$ inside single living cells specifically.

Experimental Procedures

Gene Construction—The polymerase chain reaction (PCR) was performed with KOD-Plus Neo polymerase (Toyobo,
Japan), and all of the oligonucleotide primers used in this study are listed in Table 1. DNA fragment assembly was performed using the In-Fusion HD cloning kit (Takara Bio, Japan). The NasS and NasT genes were amplified by PCR from a pUC-based clone library of *B. japonicum* (14). The cDNA of seCFP and YFP (Venus) variants with circular permutation (15) and the pCold I vector (Takara Bio) were amplified by PCR. The amplified genes were assembled to obtain pCold_CFP, pCold_CFP-NasT, and pCold_NasS-YFP for expression in *Escherichia coli*. CFPs and CFP-NasT were expressed as N-terminal His-tags tagged constructs, whereas NasS-YFP had a His tag added at its C terminus. PCR-based mutagenesis and QuikChange (Stratagene) were used to construct mutants of seCFP (A206K) and NasS and NasT (H145A), respectively. The genes CFP-NasT and NasS-Venus (cp195) were cloned into a pFLAG-CMV-1 vector (Sigma) to obtain pCMV_sNOOOpy, which was used for mammalian expression. In pCMV_sNOOOpy, the FLAG sequence was replaced by the nuclear export signal sequence of HIV Rev (LPPLERLTL), and the genes CFP-NasT and NasS-Venus_cp195 were arranged in tandem by self-processing 2A peptides.

**Purification of Proteins**—The proteins CFP, CFP-NasT, NasS-YFP, GST-tagged NasT, and His-tagged NasS were expressed and purified from *E. coli* following the same procedures as described previously (13). Appropriate fractions were dialyzed against 10 mM HEPES, pH 8.0. The homogeneity of purified proteins was established by SDS-PAGE analysis. The protein concentrations were determined using A_{280} and a molar extinction coefficient of 32,500 M⁻¹ cm⁻¹ for CFP and CFP-NasT (16) and using A_{280} and a molar extinction coefficient of 84,000 M⁻¹ cm⁻¹ for NasS-YFP (17). The protein concentrations of GST-tagged NasT and His-tagged NasS were determined by the BCA protein assay kit (Pierce) using bovine serum albumin as a standard.

**Characterization of the sNOOOpy System**—The fluorescence of the sNOOOpy system was investigated in 100 mM HEPES, pH 8.0, and 10 mM KCl using an FP-8000 spectrofluorometer (Jasco) at 25°C. To obtain the fluorescence spectra, CFP was excited with 410 ± 10 nm light, and emission from 450 to 600 nm was scanned. The NasS-NasT binding assay was performed by using multiwell plates on a TECAN Spark 10M (excitation filter, 405 nm; emission filter, 405 ± 10 nm light, and emission from 450 to 600 nm). Emission with various concentrations of NasS-YFP or CFP-NasT was measured as described for the SUMO1 and Ubc9 interaction (18), with some modifications. The FRET emission was fitted using KaleidaGraph (Synergy software) with a single-site binding model.

**Titration analyses** were performed by FRET/CFP ratios against [NO₃⁻] or [NO₂⁻] (square brackets denote concentration of proteins/ligands). Plots were fitted with Equation 1,

$$
R = R_{\Delta NasT} + (R_0 - R_{\Delta NasT}) \times \frac{K_{0^n}}{K_{0^n} + [NO_3^-]^{p}}
$$

(1)

where \( n \) is the Hill coefficient, \( K_0 \) is a [NO₃⁻] or [NO₂⁻] dissociating half of NasST; \( R_0 \) is initial FRET/CFP ratio; \( R_0 \) is initial FRET/CFP ratio in the anion-free condition, and \( R_{\Delta NasT} = R_{\Delta NasT} \) is FRET/CFP ratio of NasS-YFP with CFP, respectively.

**Cell Culture and Microscopy**—HeLa cells were obtained from Dr. Takeharu Nagai (Osaka University, Japan) and were cultured in MEM (Nacalai, Japan) containing 5.5 mM glucose supplemented with 10% fetal bovine serum (Sigma). Cells were transfected with pCMV-sNOOOpy using Lipofectamine 2000.

### Table 1

| Amplified gene/vector | Forward | Reverse |
|-----------------------|---------|---------|
| pCold_CFP, pCold_CFP-NasT | 5'-tag tga act tct gcc taa aag c-3' | 5'-cat atg cct acc ttc gat atg c-3' |
| pCold_CFP | 5'-cga agg tag gca tat gtt gac caa ggg c-3' | 5'-gca gat acc tat ctc tag ace tgg c-3' |
| seCFP (pCold_CFP) | 5'-cag agg tag gca tat ggt gac caa ggg c-3' | 5'-cag cta gca ctc gct gcg c-3' |
| NasT | 5'-cat cga cga gcc cta cat ctc gcg c-3' | 5'-ctt tta cca aga gat tac cta ttc cag cat ctc cga c-3' |
| pCold_NasS-Venus | 5'-cag cac cac cac cac cac tag gta act c-3' | 5'-ggt gta tta cct c-3' |
| pCold_vector | 5'-cag mot atc cta cag gcc acc gcg c-3' | 5'-gge ctt cca gcc acc c-3' |
| Venus | 5'-ggt cgc tgg aag gcc atg gtc aag gcc cgc c-3' | 5'-gac tca atg gtc ggt tgg tgg tgg tgg tgg tgg tgg ggc gac gcc gcg c-3' |
| Venus_cp50 | 5'-ggt cgc tgg aag gcc acc gac aag cag c-3' | 5'-gac tca atg gtc ggt tgg tgg tgg tgg tgg tgg tgg ggc gac gcc gcg c-3' |
| Venus_cp157 | 5'-ggt cgc tgg aag gcc ggc aag cag gcc c-3' | 5'-gac tca atg gtc ggt tgg tgg tgg tgg tgg tgg tgg ggc gac gcc gcg c-3' |
| Venus_cp173 | 5'-ggt cgc tgg aag gcc ggc aag cag gcc c-3' | 5'-gac tca atg gtc ggt tgg tgg tgg tgg tgg tgg tgg ggc gac gcc gcg c-3' |
| Venus_cp229 | 5'-ggt cgc tgg aag gcc gcg ctc cag gcc cgc acc c-3' | 5'-gac tca atg gtc ggt tgg tgg tgg tgg tgg tgg tgg ggc gac gcc gcg c-3'

*To construct pCMV_sNOOOpy, a pCMV_2A peptide was primarily constructed. The genes in the order of seCFP and YFP genes were amplified by PCR from a pUC-based clone library of *B. japonicum* (14). The cDNA of seCFP and YFP (Venus) variants with circular permutation (15) and the pCold I vector (Takara Bio) were amplified by PCR. The amplified genes were assembled to obtain pCold_CFP, pCold_CFP-NasT, and pCold_NasS-YFP for expression in *Escherichia coli*. CFPs and CFP-NasT were expressed as N-terminal His tags tagged constructs, whereas NasS-YFP had a His tag added at its C terminus. PCR-based mutagenesis and QuikChange (Stratagene) were used to construct mutants of seCFP (A206K) and NasS and NasT (H145A), respectively. The genes CFP-NasT and NasS-Venus (cp195) were cloned into a pFLAG-CMV-1 vector (Sigma) to obtain pCMV_sNOOOpy, which was used for mammalian expression. In pCMV_sNOOOpy, the FLAG sequence was replaced by the nuclear export signal sequence of HIV Rev (LPPLERLTL), and the genes CFP-NasT and NasS-Venus_cp195 were arranged in tandem by self-processing 2A peptides.*
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(Life Technologies, Inc.). Then, cells were transferred to a glass-bottom dish (0.17-mm thickness, MatTek) coated with type I-C collagen (Nitta Gelatin, Japan). Cells cultured in phenol red-free DMEM were subject to imaging experiments 40–72 h after transfection.

The cells were maintained on a Ti-E inverted microscope (Nikon Corp., Japan) at 37 °C in a humidified atmosphere containing 5% CO₂ using a stage-top incubator (Tokai Hit, Japan) and were visualized through a Plan Apo 40×, 0.95 numerical aperture, dry objective lens (Nikon). The filters used for dual-emission ratio imaging of sNOOOpy were purchased from Semrock (Rochester, NY) and included an FF01-438/24 excitation filter, an FF01-483/32 for CFP and an FF01-542/27 for YFP. Cells were illuminated using a xenon lamp through 25 and 12.5% neutral density filters. Fluorescence emissions from sNOOOpy were imaged using a scientific CMOS camera (Zyla 4.2, Andor Technologies). CFP and YFP images were obtained by alternating the emission filters with a filter exchanger. The exposure times were 200 ms for CFP and YFP images. The microscope system was controlled by NIS-Elements software (Nikon). Image analysis was performed using MetaMorph software (Molecular Devices). The YFP/CFP emission ratios were calculated by dividing YFP intensity by CFP intensity within a region of interest in a cell.

**Results**

**Construction of the NO₃⁻/NO₂⁻ Biosensor, sNOOOpy**—We invented an intermolecular FRET-based NO₃⁻/NO₂⁻ biosensor composed of two proteins, seCFP linked with the N terminus of NasT (19) and YFP (Venus) (Fig. 1C (20, 21). As a preliminary step in the development, we attempted to find the best combination of the two classes of fluorescent proteins fused with NasT and NasS to improve the dynamic range and signal intensity. Among the combinations summarized in Fig. 1D, the FRET signal of the protein pair composed of CFP-NasT and NasS-Venus_cp195 (a circularly permuted Venus having the 195th amino acid as its N terminus (15)) showed the highest increase in the formation of the NasS-NasT complex (NasST) and the largest change from the addition of NO₃⁻. Therefore, this protein pair was subjected to characterization and further development.

When 1 μM CFP-NasT was mixed with 1 μM NasS-YFP, the FRET/CFP emission ratio increased to 1.8 as assessed by the emission ratio of 527/475 nm, a 3.6-fold increase from the CFP and NasS-YFP protein pairs (Fig. 1, E and F, left panels). Additions of NO₃⁻ and NO₂⁻ abrogated the increase in the emission in a concentration-dependent manner, although these anions showed no effects on emissions of fluorescence proteins (Fig. 1, E and F). The change in FRET signal exhibited a high selectivity for NO₃⁻ and NO₂⁻. Among nine oxoanions summarized in Fig. 2, only NO₃⁻ and NO₂⁻ reduced the FRET ratio. Furthermore, such reductions induced by NO₃⁻ were not interrupted by the presence of the other oxoanions. Thus, the indicator system can specifically detect the change in NO₃⁻ and NO₂⁻ levels as the FRET signal changes, which is caused by association and dissociation of NasS-YFP and CFP-NasT as designed in Fig. 1B. We termed the generated indicator system composed of CFP-NasT and NasS-YFP as sNOOOpy senor for NO₃⁻/NO₂⁻ in physiology. sNOOOpy-cp195, composed of CFP-NasT and NasS-Venus_cp195, was adopted as the wild-type sNOOOpy (sNOOOpy WT), and the sNOOOpy variants constructed in this study are summarized in Table 2.

**In Vitro Characterizations of NasS-NasT Interaction by sNOOOpy**—First, we characterized the protein interaction between CFP-NasT and NasS-YFP. Titration of 1 μM CFP-NasT with NasS-YFP showed that apparent dissociation constant (K_{d}) between CFP-NasT and NasS-YFP is estimated to be 0.13 μM (Fig. 3, A and B). The FRET ratio of sNOOOpy was decreased by titrate in increasing unlabeled [NasS], indicating that the interaction between NasS and NasT is reversible (Fig. 3C).

Next, we focused on the NO₃⁻/NO₂⁻-sensing mechanism of NasST at the molecular level. In rhizobial cell function, NO₃⁻/NO₂⁻ induce dissociation of NasST by binding to NasS. Therefore, we inferred that NO₃⁻/NO₂⁻ can be regarded as a competitive inhibitor that competes with NasT for binding to NasS (Fig. 3D). Fig. 3, E and F, shows titration of 1 μM NasS-YFP with CFP-NasT in the presence of various [NO₃⁻]. Although curve-fitting analyses based on the single-site binding model failed because of the progressive decrease of FRET emission at high [CFP-NasT], the inhibitory effects of NO₃⁻ at each [CFP-NasT] were comparable (Fig. 3G). NO₂⁻ decreased FRET emission at low [CFP-NasT], whereas those at high [CFP-NasT] were recovered to the levels of NO₃⁻-free conditions. These results supported that NO₃⁻/NO₂⁻ inhibit NasS-NasT formation competitively.

**Sensitivity of the sNOOOpy System in Vitro**—Prior to further characterizations, we have prepared sNOOOpy(H145A), which is composed of a NasS(H145A) mutant that was inferred to form a NO₃⁻-binding site based on the NO₃⁻-binding structure of the NO₃⁻-binding protein NrtA (Fig. 4A) (22). Next, we characterized the sensitivity of the sNOOOpy system. In this study, we used K_{d} values, the values at which the [NO₃⁻] or [NO₂⁻] dissociates half of the NasST, as the sensitivity determinant of the sNOOOpy system. Because the K_{d} value corresponds to the half-maximal inhibition concentration (IC_{50}) inhibiting NasST formation in the competitive model as shown in the Fig. 3D, the K_{d} values were determined by curve-fitting analyses using the Hill equation. Fig. 4B shows the FRET/CFP ratio from titration with NO₃⁻ and NO₂⁻. When 1 μM each of CFP-NasT and NasS-YFP was used, a micromolar [NO₃⁻] decreased the FRET ratio, which is in good agreement with the affinity reported for NasST from Paracoccus denitrificans (K_{D} = 15 μM) (11). Under our in vitro assay conditions, the K_{d} values for NO₃⁻ and NO₂⁻ were estimated to be 39.5 and 256 μM, respectively. The FRET signal of sNOOOpy was not reduced upon the addition of <10 mM NO₃⁻/NO₂⁻, indicating that NasS(H145A)-YFP and CFP-NasT form an extremely stable complex and lost NO₃⁻/NO₂⁻ responsiveness.

Elevated K_{d} values corresponding to an increase in the [CFP-NasT] were observed (Fig. 4C). This characteristic in the sensitivities reflects that sNOOOpy is based on an intramolecular FRET system, where CFP-NasT and NO₃⁻ competitively binds to NasS-YFP as shown in Fig. 3D. In a canonical competitive model, IC_{50} values depend on the substrate concentration, IC_{50} =
where IC50, Ks, and Ks correspond to Kd, CFP-NasT, and Ks-NO3 in Fig. 3D, respectively. From the linear fit based on the equation, Ks-NO3 and Ks-NO3/Ks were calculated to be 6.0 M and 39, respectively (Fig. 4D). Estimated Ks value, 0.15 M, was in good agreement with the value determined by titration of CFP-NasT with NasS-YFP, 0.13 M, as shown in Fig. 3B and Equation 2.

\[
E = E_{\text{max}} \times \left( \frac{K_{\text{ST}} + [S]_{\text{total}} + [T]_{\text{total}}^* - \sqrt{K_{\text{ST}} + [S]_{\text{total}} + [T]_{\text{total}}^* + 4 \times [S]_{\text{total}} \times [T]_{\text{total}}}}{2} \right)
\]

(Eq. 2)

where E is the fluorescent emission at 535 nm; Ks is dissociation constant between CFP-NasT and NasS-YFP; [S]total is the total

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**FIGURE 1.** FRET-based NO3/NO2 probes, sNOOOpy. A, proposed model of a two-component regulatory system composed of NasS-NasT. NasS plays a negative regulatory role by interacting with NasT. In the presence of NO3 or NO2, the putative RNA-binding protein NasT is released from NasS and acts as a transcription anti-terminator that binds the leader sequence in mRNA, preventing hairpin formation and allowing complete transcription of the genes. B, schematic drawing of the sNOOOpy system. CFP and YFP (Venus) are connected with NasT and NasS, respectively. In the NO3- or NO2-bound form, dissociation of the two proteins separates the two fluorescent proteins, which decreases FRET efficiency. F, I, fluorescence intensity. C, schematic diagram of sNOOOpy proteins, CFP-NasT and NasS-YFP (Venus_cp195). D, FRET/CFP ratio changes in NasS fused with different Venus variants. Fluorescent emissions of NasS fused with Venus variants (1 μM) were measured in the presence of CFP (1 μM) (open square), CFP-NasT (1 μM) (closed square), or CFP-NasT with 2 μM of NO3 (gray square). The labels 50, 157, 173, 195, and 229 indicate circularly permuted Venus having the 50th, 157th, 173rd, 195th, and 229th amino acid as its N terminus, respectively. E and F, fluorescence emissions of sNOOOpy. Fluorescence was measured by excitation with 410 nm (left and middle) or 475 nm (right) light at various concentrations of NO3 (E) and NO2 (F) at 25 °C using protein pairs of 1 μM each of CFP-NasT + NasS-YFP (left), or CFP-NasT + His-tagged NasS (middle), or GST-tagged NasT + NasS-YFP (middle and right) in 100 mM HEPES-NaOH, pH 8.0, and 10 mM KCl. Emissions of sNOOOpy-ΔNasT, which is composed of CFP and NasT-YFP, are shown as broken lines.
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NasS-YFP; and [T]_{total} is the total CFP-NasT ( = 1 μM) concentrations, respectively. $K_{ST}$ was calculated by the fit.

The time course of FRET ratio changes at 20–100 μM NO$_3^-$ revealed that the rates of NO$_3^-$ binding ($k_{on}$) and dissociation ($k_{off}$) were determined based on the first order fitting to be $2.3 \times 10^{-3} \, \mu M^{-1} \, s^{-1}$ and 0.10 s$^{-1}$, respectively (Fig. 5, A and B). Thus, sNOOOpy system can detect [NO$_3^-$] changes on a time scale of seconds.

The fluorescence emission ratio was almost invariant from pH 7.0 to 8.5 and over a temperature range of 25 to 40 °C, suggesting that the sensitivity of sNOOOpy will not be affected by small fluctuations in pH and temperature (Fig. 5, C and D). The $K_D$ value for NO$_3^-$ at 37 °C (37.9 μM) is almost identical to that at 25 °C (39.5 μM) (Fig. 5E). Thus, these results showed that we can quantify [NO$_3^-$] in a range of 1–1000 μM by sNOOOpy under standard in vitro assay conditions.

**Imaging of NO$_3^-$/NO$_2^-$ Levels Inside Single Living Cells**—Next, we visualized the NO$_3^-$/NO$_2^-$ levels inside of a single living HeLa cell expressing sNOOOpy proteins. First, we constructed a mammalian expression plasmid for sNOOOpy, pCMV-sNOOOpy (Fig. 6A), in which cDNAs coding for CFP-NasT, a self-processing 2A peptide, and NasS-YFP were arranged in

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**FIGURE 2. Specificities of the sNOOOpy system.** FRET/CFP ratio of sNOOOpy in the presence of 0 (gray), 100 μM (green), 10 mM (red), and 100 μM NO$_3^-$ with 10 mM of each anion (yellow) is shown. Solid and broken lines indicate FRET/CFP ratio of sNOOOpy with 100 μM NO$_3^-$ and sNOOOpy-ΔNasT, which is composed of CFP and NasT-YFP, respectively.

**TABLE 2**

sNOOOpy variants constructed in this study

| Name               | Component proteins | Characteristics in vitro |
|--------------------|--------------------|--------------------------|
| sNOOOpy-ΔNasT      | CFP, NasS-Venus_cp195 | Negative control          |
| sNOOOpyWT          | CFP-NasT, NasS-Venus_cp195 | Wild-type sNOOOpy that subjected to characterizations |
| sNOOOpyH145A       | CFP-NasT, NasS(V145A)-Venus_cp195 | Form stable dimer that is not dissociated by NO$_3^-$/NO$_2^-$ |

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**FIGURE 3. Characterizations of NasS-NasT interaction by sNOOOpy system in vitro.** A, fluorescence emissions at 535 nm from the NasS-NasT binding assay using multiwell plates on a TECAN Spark 10M (excitation filter, 405 ± 10 nm; emission filter, 535 ± 10 nm). Emission of 1 μM CFP-NasT is almost identical to that at 25 °C (39.5 μM) (Fig. 5E). Thus, these results showed that we can quantify [NO$_3^-$] in a range of 1–1000 μM by sNOOOpy under standard in vitro assay conditions.

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FIGURE 4. Sensitivities of sNOOOpy in vitro. A, NO$_3^-$-binding site structure of NrtA. Bound NO$_3^-$ is shown as a sphere model. Side chain of the His-196 that forms direct interaction with NO$_3^-$ is shown. The residue name and numbers in parentheses indicate the corresponding residue in NasS. This image was prepared using PyMOL (DeLano Scientific, Palo Alto, CA). Amino acid sequence alignment between NasS and NrtA are shown in the bottom panel. The residues involved in binding of a nitrate are indicated by blue dots. The sequences were aligned with Clustal Omega (36), and this image was prepared using ESPript (37). B, FRET/CFP ratio of sNOOOpy WT (WT) and sNOOOpy H145A (H145A) plotted against increasing concentrations of NO$_3^-$ (red) and NO$_2^-$ (blue). Values in parentheses indicate the $K_d$ values for NO$_3^-$ (red) and NO$_2^-$ (blue), the values at which the [NO$_3^-$] or [NO$_2^-$] dissociates half of the NasST. Concentrations of 1 µM sNOOOpy protein, composed of equal concentrations of CFP-NasT and NasS-YFP, were used. The $K_d$ values were calculated by curve-fitting analysis using the standard Hill equation. C, FRET/CFP ratios at various concentrations of [CFP-NasT] were plotted against increasing [NO$_3^-$]. Concentrations of 1 µM NasS-YFP and 0.5–2.0 µM of CFP-NasT were used. D, $K_d$ values were plotted against [CFP-NasT] and fitted by equation: $K_d = (1 + [CFP-NasT]/K_{ST}) \times K_{S-NO3}$. From linear fit to the plot, $K_{S-NO3}$, $K_{ST}$, and $K_{S-NO3}/K_{ST}$ were calculated as 6.0 and 0.15 µM and 39, respectively.

FIGURE 5. Characterizations of sNOOOpy. A, time course of the FRET/CFP ratio change in the presence of various concentrations of NO$_3^-$, B, apparent rate constants ($k_{app} = k_{on}[NO_3^-] + k_{off}$) were plotted against [NO$_3^-$]. From linear fit to the plot, $k_{on}$ and $k_{off}$ were calculated as 0.0023 s$^{-1}$ and 0.10 s$^{-1}$, respectively. C, pH and $T$ temperature dependences of sNOOOpy. The values of $K_d$ and $R_{NSat}$ indicate the emission ratio of control (in the anion-free buffer) and that of sNOOOpy-$\Delta$NasT, respectively. The FRET/CFP ratio at 25 °C at 0, 10, 100, and 1000 µM NO$_3^-$ in the pH range of 6.0–9.0 are shown. The buffer contained 100 mM MES-NaOH (pH 6.0–7.0, open circles) or HEPES-NaOH (pH 7.0–9.0, closed circle). $E$, kinetic analyses of sNOOOpy WT in vitro at 25 °C (black) and 37 °C (red). FRET/CFP ratio was plotted against increasing concentrations of NO$_3^-$, Concentrations of 1 µM sNOOOpy protein, which was composed of equal concentrations of CFP-NasT and NasS-YFP, were used.

In the HeLa cells expressing sNOOOpy WT, although up to 0.1 mM NO$_3^-$ induced no obvious changes of the FRET/CFP ratio (Fig. 6D), NO$_3^-$ at a concentration of 0.3 mM induced a significant and rapid decrease in the FRET/CFP ratio, and the ratio was decreased about 50% by addition of 3 mM NO$_3^-$ (Fig. 6E). The HeLa cells containing sNOOOpy WT were less sensitive to NO$_2^-$ compared with NO$_3^-$, but apparent changes in the FRET/CFP ratio were detectable at 1 mM NO$_2^-$ (Fig. 6C, middle panel). The HeLa cells with sNOOOpy H145A, which formed stable NasST complexes and lost NO$_3^-$/NO$_2^-$ responsiveness, were insensitive to a change in ambient [NO$_3^-$] (Fig. 6C, bottom panel). It should be noted that the response of sNOOOpy to NO$_3^-$ is reversible. Fig. 7 and supplemental Movie 2 show the
time course of the FRET/CFP ratio when the medium [NO₃⁻/H₁₁₀₀₂] was alternately changed. The FRET/CFP ratio was reduced from 1.4 to 0.8 (40% decrease relative to that in 0 mM) when the cells were cultured in DMEM containing 1 mM NO₃⁻/H₁₁₀₀₂ for 15 min. Ten minutes after an exchange of the medium to a NO₃⁻/H₁₁₀₀₂-free one, the FRET/CFP ratio had recovered to the initial level. Rates of FRET ratio changes in the cells were determined based on the difference of the ratio at two points of time (Fig. 7B). Rates of changes were estimated to be 0.0011–0.0034 (average 0.0022; 18 cells) by addition of 1 mM [NO₃⁻/H₁₁₀₀₂] to DMEM and 0.0011–0.0022 (average 0.0015; 18 cells) by removal of NO₃⁻/H₁₁₀₀₂.

Discussion

Biosensor Employing a Bacterial Environmental Response System—NasS and NasT in the root nodule bacterium B. japonicum are involved in a two-component response system to environmental [NO₃⁻] and regulate protein levels related to NO₃⁻ assimilation (13). We exploited the change in their association-dissociation behavior depending on [NO₃⁻] to develop a NO₃⁻ biosensor. sNOOOpy is the first FRET-based biosensor derived from a bacterial environmental response system. Typically, bacterial two-component regulatory systems comprise a sensor histidine kinase and its cognate response regulator (25), and their functions are controlled by phosphorylation levels of the response regulator catalyzed by histidine kinase in response to environmental stimuli. However, the two-component system of NasS-NasT is regulated without phosphorylation, meaning the reaction is energetically reversible. The sNOOOpy system does not require any component, such as ATP or metal ions, for detection of the [NO₃⁻] change, and it can furthermore detect both an increase and decrease of [NO₃⁻]. These results provide evidence that a potential biological function of the
NasS-NasT two-component system is to respond to not only the increase but also the decrease of cellular \([\text{NO}_3^-]\), allowing the bacteria to presumably suppress the protein transcription level. An NasS-NasT-like two-component system, AmiC and AmiR, that regulates protein levels involved in the catabolic degradation of aliphatic amides was also found in *Pseudomonas aeruginosa* (26, 27). When considered with the development of the sNOOOpy system from NasS-NasT, we suggest the possibility of an aliphatic amide sensor derived from AmiC-AmiR.

**Sensitivity of the sNOOOpy System in Vitro and in Vivo**—NO\(_3^-\)/NO\(_2^-\) are reported to be in micromolar levels in tissues, blood, and plasma (3–50 \(\mu M\)) (28) and in millimolar levels in urine (8). Therefore, to monitor the change in \([\text{NO}_3^-]\)/\([\text{NO}_2^-]\) levels in physiological processes, indicators must have submicromolar to submillimolar sensitivity. When a protein concentration of 1 \(\mu M\) each of CFP-NasT and NasS-YFP is used, the sNOOOpy system can detect a change in \([\text{NO}_3^-]\) in the micromolar range using our *in vitro* assay conditions. This detection limit of the sNOOOpy system is similar to that of the Griess method.

To determine whether the sNOOOpy system can work in mammalian cells, we subjected cells harboring sNOOOpy proteins to medium containing physiological concentrations of \([\text{NO}_3^-]/\text{NO}_2^-\) (0.01–10 mM). Although little is known about influx and efflux of \([\text{NO}_3^-]\) from mammalian cells, the \([\text{NO}_3^-]\) influx and characterization of sialin, a 2\(\text{NO}_3^-/\text{H}^+\) cotransporter, were investigated only in human submandibular gland cell line cells (9). In the extracellular solution containing physiological concentrations of \([\text{NO}_3^-]\) (0.05–0.5 mM), a patch clamp method detected an anion current derived from \([\text{NO}_3^-]\) influx into human submandibular gland cell line cells at low pH conditions. Recently, the cell bank found that human submandibular gland cell line cells originated from HeLa cells. Therefore, in this study, we exploited the HeLa cells for live cell \([\text{NO}_3^-]/\text{NO}_2^-\) imaging. Although no obvious changes were observed up to 0.1 mM \([\text{NO}_3^-]\), the FRET ratio of sNOOOpy was changed in response to 0.3–3 mM \([\text{NO}_3^-]\) in the medium even at a neutral pH condition.

Although >90% of CFP-NasT and NasS-YFP were dissociated in the presence of 1 mM \([\text{NO}_3^-]\) in *in vitro* assay conditions, the FRET ratios in the HeLa cells showed obvious changes at levels up to 3 mM in the medium. Furthermore, the rate of FRET/CFP ratio changes *in vitro* is 0.2 s\(^{-1}\) by addition of 40 \(\mu M\) \([\text{NO}_3^-]\) to assay conditions, which was markedly faster than that in HeLa cells, 0.002 s\(^{-1}\) by addition of 1 mM \([\text{NO}_3^-]\) to the medium. Here, we consider some of the reasons for the differences of the sNOOOpy system between *in vitro* and *in vivo*. One possibility is that the cellular \([\text{NO}_3^-]\) is not raised to the same level as that in the medium, so concentrations in the medium might not be accurately reflected in the HeLa cells. Another possibility is that the sensitivity of the sNOOOpy is affected by some cellular environmental factors. Our results showed that excess of CFP-NasT protein increases \(K_D\) values caused by an intermolecular FRET system. Such protein concentration dependence of sNOOOpy may be avoided by developing sNOOOpy to be an intramolecular system.

Among NasS-like proteins, the crystallographic study of NrtA, \([\text{NO}_3^-]/\text{[NO}_2^-\)]-binding protein from cyanobacteria, has been reported (22), and residues involved in \([\text{NO}_3^-]\) binding were identified. In this study, we constructed sNOOOpy\(^{H145A}\), which comprises the NasS\(^{H145A}\) mutant, and we revealed an important role of the residue for \([\text{NO}_3^-]/\text{NO}_2^-\) responsiveness by the sNOOOpy system. However, structural bases of the interaction of NasS with NasT are still unclear. Crystallographic studies are desired for further improvement in sensitivity and specificity of the sNOOOpy system.

Furthermore, strong base fluorescence emission might limit the detection sensitivity. Therefore, it might be possible to further improve the FRET signal of sNOOOpy by using a polarized fluorescence excitation and detection technique (29).

**Insight for Future Applications of the sNOOOpy System**—As \([\text{NO}_3^-]/\text{NO}_2^-\) are accepted as stable reservoirs that can be
sNOOOpy, a Sensor for Nitrate/Nitrite in Living Cells

reduced to bioactive NO through the “NO$_3^-$-NO$_2^-$-NO pathway,” the potential benefits of NO$_3^-$/NO$_2^-$ in the health field have received much attention. For example, recent prospective epidemiologic studies have shown that green leafy vegetables protect against coronary heart disease and ischemic strokes (30, 31). Many researchers explained these effects using the biologically plausible hypothesis that NO$_3^-$/NO$_2^-$ in the diet can provide substrates for NO, which results in vasodilation, decreased blood pressure, and supported cardiovascular function (32–34). Thus, NO/NO$_3^-$/NO$_2^-$ studies have advanced rapidly in the last decade, and the possibility that these anions can be used as therapy for human diseases (e.g. myocardial infarction, stroke, solid organ transplantation, and sickle cell disease) has been suggested (3, 6, 35). Furthermore, Tang et al. (5) suggested that determining endogenous NO$_3^-$/NO$_2^-$ levels is expected if these anions can be used as diagnostic biomarkers of disease or treatment regimens. Unfortunately, despite the increased attention to the NO$_3^-$-NO$_2^-$-NO pathway, no other methodology like that of the Griess reaction has been widely used.

We demonstrate that the sNOOOpy system is a NO$_3^-$/NO$_2^-$-specific biosensor that enables us to visualize both the increase and decrease in NO$_3^-$/NO$_2^-$ levels in mammalian cells (Fig. 7C). In particular, because the sNOOOpy system is applicable to living cells, which is unique compared with conventional measurement methods, sNOOOpy makes it possible to observe the intracellular dynamics of NO$_3^-$/NO$_2^-$ both in situ and in real time. The sNOOOpy system paves the way for the elucidation of NO/NO$_3^-$/NO$_2^-$ biology. Finally, we refer to the potential usefulness of the sNOOOpy system for applications in bacteria and plants. NO$_3^-$/NO$_2^-$ serves as an essential nutrient for plant growth and survival, because of its involvement in several crucial biological functions of plants (e.g. tissue development and immune systems). The visualization of NO$_3^-$/NO$_2^-$ dynamics in the physiological actions of plants by the sNOOOpy system has the potential to contribute to the elucidation of fundamental and applied mechanisms of plant life.

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