A Fluorescence Resonance Energy Transfer-Based Analytical Tool for Nitrate Quantification in Living Cells

Urooj Fatima,* Fuad Ameen, Neha Soleja, Parvez Khan, Abobakr Almansob, and Altaf Ahmad*

ABSTRACT: Nitrate (NO$_3^-$) is a critical source of nitrogen (N) available to microorganisms and plants. Nitrate sensing activates signaling pathways in the plant system that impinges upon, developmental, molecular, metabolic, and physiological responses locally, and globally. To sustain, the high crop productivity and high nutritional value along with the sustainable environment, the study of rate-controlling steps of a metabolic network of N assimilation through fluxomics becomes an attractive strategy. To monitor the flux of nitrate, we developed a non-invasive genetically encoded fluorescence resonance energy transfer (FRET)-based tool named “FLIP-NT” that monitors the real-time uptake of nitrate in the living cells. The developed nanosensor is suitable for real-time monitoring of nitrate flux in living cells at subcellular compartments with high spatio-temporal resolution. The developed FLIP-NT nanosensor was not affected by the pH change and have specificity for nitrate with an affinity constant ($K_a$) of $\sim$5 $\mu$M. A series of affinity mutants have also been generated to expand the physiological detection range of the sensor protein with varying $K_a$ values. It has been found that this sensor successfully detects the dynamics of nitrate fluctuations in bacteria and yeast, without the disruption of cellular organization. This FLIP-NT nanosensor could be a very important tool that will help us to advance the understanding of nitrate signaling.

INTRODUCTION

Nutrients are indispensable elements required for the growth and development of all living organisms. Plants acquire minerals from the soil through the root system. Each type of plant has a minimum required level of nutrients and an optimum nutrient range.$^1$ Plants exhibit nutrient deficiency symptoms below the minimum required nutrient level, and at excessive nutrient uptake, they show weak growth due to toxicity.$^2$ Therefore, a balanced supply of nutrients is essential in increasing crop yields. Nitrogen (N) is one of the essential macronutrients, a major component of biomacromolecules, like protein, nucleic acid, chlorophyll, and vitamins. In the agricultural field, nitrate (NO$_3^-$) is supplied in the form of urea as a source of N. The nitrate (NO$_3^-$) concentration is making a predominant form of N available to cereal crops. Absorbed nitrate from the root is mobilized to other organs contributed by nitrate transporters (NRT1 and NRT2). To be incorporated into amino acids, NO$_3^-$ assimilation into amino acids was reduced to nitrite (NO$_2^-$) in the cytosol by nitrate reductase, and NO$_2^-$ is then reduced to NH$_4^+$ in plastids or chloroplasts by a nitrite reductase.$^3$ In addition to being an essential nutrient, the nitrate also serves as a signaling molecule that influences both the metabolic and developmental processes of plants and directs the expression of a wide range of genes.$^4,5$ Nitrate induces genes of its assimilation pathway,$^6$ breaks seed dormancy,$^7$ and regulates the root system architecture.$^8$

The sustenance of the maximal crop yield depends critically on massive applications of N fertilizers (>120 Mt) applied to the agricultural field globally, only 25—50% is taken up by plants.$^8$ Excess of N is lost into the environment by leaching, denitrification, and volatilization.$^9$ Globally, the food demand is expected to increased by 40% by 2030 to feed the growing population, which is predicted to be raised by at least 25% by 2050.$^10$ Significant challenges are to improve crop yield to feed the teeming population but with a minimum impact on the environment and human health caused by excess unutilized nitrate.

For a reason, to protect human health and the environment, it is necessary to develop a useful tool for the detection of NO$_3^-$ in live cells. Therefore, this non-invasive tool would help us to gain insights into the rate-controlling and regulated step, especially in the context of flux regulation. Metabolic flux may be estimated by recording mass transfer or by using a tracer. The process of nitrate assimilation needs to be well-coordinated in response to nitrogen supply and demand of living cells. The advent of a broad range of genomic technologies and genetic tools has revolutionized cell and molecular biology that enables essential advancement in our
understanding of the mechanisms that is controlling of nitrate and other N nutrient responses.

Varied detection strategies have been reported in literature based on anion chromatography,11 radioactive isotope $^{15}$N,12 Griess method,13 and microelectrode, and $^{13}$Cs nuclear magnetic resonance.14 However, these methods either require the fraction of tissues or lack high temporal or spatial resolution. Moreover, none of the approaches permits real-time monitoring of nitrate levels in living cells.

The nitrate sensor and signaling pathway are yet to be developed to analyze the regulatory steps in the metabolic network in real-time
1vivo. This would later help us to decipher at what concentration NO$_3^-$ acts as a rate-controlling of regulatory steps in nitrate assimilation pathway but below that of toxic levels.

To provide exquisitely detailed information about the comprehension of the regulation of nitrate homeostasis and the role of NO$_3^-$ in (intracellular) signal transduction, FRET-based genetically encoded non-invasive tools may be useful. It allows imaging of NO$_3^-$ concentrations at the subcellular level in the living cells and in real-time that trace the dynamic nitrate concentration at the nanoscale.

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The FRET combined with the ratio-metric fluorescent probe is best suited for NO$_3^-$ sensing purpose because it can detect NO$_3^-$ concentration at subcellular compartments in a nondestructive manner. The efficiency of FRET is primarily controlled by the spectral overlap between the donor and acceptor pairs that allows FRET to occur. The FRET is a distance, and angle-dependent phenomenon permitted particle interactions within a range of Förster distance (1–10 nm).15 The FRET relies on the direct excitation of the acceptor by a donor emission wavelength in a nonradiative fashion when the distance separating between them is within the Förster range.16 Thus, a cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) act as a donor and acceptor, respectively, which satisfy a prerequisite for the design of the FRET-based genetically encoded nitrate nanosensor.

Out of consideration of circumstances related to nitrate, we have developed a genetically encoded FRET-based nanosensor for nitrate named “FLIP-NT” by the exploitation of NrtA, a periplasmic binding protein from Synechocystis sp. 6803, cyanobacteria, which are linked with CFP and YFP at N- and C-termini, respectively. This nanosensor was able to perform real-time monitoring of nitrate in living cells.

■ RESULTS

Molecular Docking. NrtA protein consists of two domains, which are linked by a C-shape clamp in which the nitrate binding site is present (Figure 1A). The NrtA-nitrate complex was stabilized by several noncovalent interactions offered by the binding site residues of NrtA. Nitrate forms seven hydrogen bonds with amino acid residues of the NrtA protein. The oxygen atoms of nitrate offer two hydrogen bonds to Gly250 of NrtA, one with Gln155 (2.9 Å), one with Lys269 (2.8 Å), two hydrogen bonds with His196 (3.0 Å), and other with Trp102 (2.8 Å) (Figure 1D). These results indicate that nitrate binds strongly to the NrtA. The C-shaped clamp present in between the two domains provides flexibility to the overall structure of the NrtA protein, which is an important aspect of transporter proteins.

Construction of a FLIP-NT Sensor. The NrtA gene from the genomic DNA of Synechocystis sp. 6803 without a signal peptide and without stop codon was amplified and cloned into the pGEM−T Easy vector followed by TA cloning. An

Figure 1. Molecular docking studies of NrtA protein. (A) Cartoon representation of the NrtA crystal structure (PDBID: 2G29), NrtA comprises two α/β globular domains (shown in red and yellow color) and a C-clamp like domain (blue color), and the nitrate binding site is present in between the two domains. (B) 3D schematic diagram of NrtA with nitrate and binding residues in the ball-stick model. (C, D) Focused view of NrtA binding site residues and nitrate, showing respective H-bonds.
amplified CFP of ∼0.72 kb was ligated at N-terminus, and an amplified YFP of ∼0.72 kb was ligated at the C-terminus of the NrtA gene producing “CFP_NrtA_YFP” cassette in the pGEM-T easy vector. The resultant construct was confirmed by restriction digestion with Xhol and HindIII, releasing bands of ∼3.0 kb of “pGEM-T” easy vector and ∼2.6 kb of “CFP_NrtA_YFP” (Figure 2A).

Finally, CFP_NrtA_YFP construct was subcloned into the expression, pRSET-B vector at Xhol, and HindIII restriction sites. The clone in expression vector was confirmed by restriction digestion with the SpeI and HindIII, releasing a ∼4.8 kb band of intact pRSET-B_CFP_NrtA and a ∼0.72 kb band of YFP (Figure 2B).

**In vitro Analysis of a FLIP-NT Sensor.** A purified FLIP-NT sensor protein was subjected to in vitro assays. Spectral analysis of the sensor protein was performed in a spectrofluorometer to prove that the nitrate addition shows corresponding changes in the fluorescence emission intensities of CFP (donor peak) and YFP (acceptor peak) by excitation at 435 nm. In the presence of 1 μM of NO₃⁻, the emission intensity of CFP (485 nm) decreased, and the emission intensity of YFP (535 nm) increased, confirming the presence of FRET. In the absence of NO₃⁻ (control), no changes in the fluorescence emission intensities were observed specifying the absence of FRET (Figure 3).

The stability of the sensor protein for physiological applications was characterized based on the least change in dual emission intensity ratio (Em₄₃₅/Em₄₈₅) at different pH (5.0−9.0) values and buffer systems (20 mM each of Tris-Cl, PBS, and MOPS). We reported that the (Em₄₃₅/Em₄₈₅) ratio fluctuates in the case of PBS and Tris-Cl buffers. Indicating, sensor protein in these two buffers is sensitive toward the pH change. In the case of 20 mM of MOPS buffer, we observed a nonsignificant change in the fluorescence emission intensity ratio at alkaline pH (Figure 4A), indicating that there will no effect of physiological pH fluctuations on the conformation of sensor protein. Thus, sensor protein is stable and remains active at the physiological pH range in MOPS buffer. Hence, 20 mM MOPS buffer, pH 7.2 was selected for further detailed studies.

pH is a critical factor for the intracellular environment, and externally applied agents may cause a change in the pH and that is also responded by cells also. Therefore, we also recorded the fluorescence emission ratio in the absence and presence of 1 μM nitrate after diluting FLIP-NT sensor protein in 20 mM MOPS buffer. No significant change in the FRET ratio was observed in the presence and absence of nitrate indicating that sensor protein was stable at physiological pH (Figure 4B). The experiment was performed in a 96-well plate using a fluorescence microplate reader. These results suggested that the FLIP-NT sensor is stable at the physiological pH range and can be used for nitrate quantification in the physiological pH range.

To see the specificity of FLIP-NT sensor protein toward NO₃⁻, the specificity and functionality of the sensor were studied with different molecules, i.e., nitrate, sulphate, phosphate, chlorate, and bicarbonate at 0, 1, and 5 μM concentrations. The maximum fluorescence ratiometric change was observed at 5 μM NO₃⁻, whereas a low FRET ratio was observed at 1 μM NO₃⁻, indicating that the maximum binding occurs at 5 μM of NO₃⁻. While with the rest of the molecules, a minimal change in the fluorescence emission ratio was observed (Figure 5). This shows that the FLIP-NT sensor is specific for NO₃⁻ detection.

To find out the affinity constant (Kₐ) of the sensor protein, a titration analysis was conducted with varying nitrate concentrations (nM to mM). A sigmoidal curve was obtained, which
was fitted to calculate the value of $K_d$ that was approximately 5 μM. With the increasing concentration of nitrate, an increase in the fluorescence emission ratio from 1.2 to 1.8 was observed, and the saturation point of the FLIP-NT sensor protein was obtained at a 40 μM nitrate concentration (Figure 6).

**Affinity Mutants.** To investigate the dynamic range of nitrate detection by FLIP-NT sensor protein, several affinity mutants were generated (Figure 7) by inserting a point mutation in the NrtA protein construct of CFP_NrtA_YFP. The point mutations were created using site-directed mutagenesis by replacing the amino acid residues of the nitrate binding pocket. These mutants exhibited a wide range of $K_d$ for nitrate and broadened the physiological range of nitrate detection. Three variants G240Q (FLIP-NT-32), W102A (FLIP-NT-56), and Q155I (FLIP-NT-1) were created with the basic ideas of replacing the amino-acid residues with polar amino acid residues or with a bulky hydrophobic group. The fluorescence emission ratio of all three mutants in the presence of nitrate was recorded to calculate respective $K_d$ values. It was observed that the mutation of Trp to Ala at position 102 led to the highest $K_d$ value, i.e., 56 μM, and shows a lowest binding affinity for nitrate. On the other hand, a mutation in Gly to Gln at position 240 yields the $K_d$ of 32 μM. Replacement of Gln to Ile at position 155 appreciably decreases the $K_d$ value and thus showing the highest binding affinity ($K_d = 1 \mu M$) for real-time monitoring of nitrate dynamics (Table 1).

![Figure 4](image_url)  
**Figure 4.** In vitro assay of the FLIP-NT nanosensor. (A) Showing the sensitivity of sensor protein toward pH with different buffers; Tris-Cl, PBS, and MOPS buffers (pH 5.0 to pH 9.0). The maximum change in fluorescence intensity ratio was observed in the case of 20 mM MOPS buffer. (B) YFP/CFP emission ratio change was recorded in the absence and presence of 1 μM NO$_3^-$ in 20 mM, MOPS buffer. Error bars indicate a mean ± standard deviation from $n = 3$.

![Figure 5](image_url)  
**Figure 5.** Specificity of the FLIP-NT sensor protein was checked by measuring the FRET ratio with different molecules. The maximum change in the fluorescence emission ratio was obtained with 5 μM nitrate, proving that the sensor is specific toward nitrate. Error bars represent mean ± standard deviation from $n = 3$.

![Figure 6](image_url)  
**Figure 6.** Ligand binding isotherm of the nitrate sensor (FLIP-NT). The FRET ratio (YFP/CFP) change was measured at different concentrations of nitrate to get a saturation curve. Error bars indicate mean ± standard deviation from $n = 3$.

![Figure 7](image_url)  
**Figure 7.** In vitro titration curve of the affinity mutants (FLIP-NT-32, FLIP-NT-56, FLIP-NT-1 μ) compared with the wild type. Fluorescence emission ratio (YFP/CFP) change was recorded in the presence of varying nitrate concentrations. Data points are expressed as mean ± Standard deviation from $n = 3$.

| S. no | nomenclature | mutation | $K_d$ (μM) | dynamic range (μM) |
|-------|--------------|----------|------------|-------------------|
| 1     | FLIP-NT-5    | wild type | 5          | 0.5–40            |
| 2     | FLIP-NT-32   | G240Q    | 32         | 0.6–50            |
| 3     | FLIP-NT-56   | W102A    | 56         | 0.2–80            |
| 4     | FLIP-NT-1    | Q155I    | 1          | 0.7–20            |

*Nomenclature of the nanosensor is based on the name of the wild-type sensor and the numeric value of binding constants ($K_d$), a number alongside the sensor. The dynamic range represents the range of detection of nitrate between 10 and 90% saturation of the nanosensor.
Cell-Based Studies of Nitrate Uptake in Living Cells. In Prokaryotes. The bacterial cells (E. coli BL21-codon plus strain), expressing the FLIP-NT sensor, was used as a prokaryotic model to non-invasively observe the real-time intracellular dynamics of nitrate in living cells. In this study, we used live-cell suspension cultures of a bacteria expressing FLIP-NT nanosensor for confocal imaging (Figure 8). Uptake of nitrate in bacteria was observed for 13 min specifying the FRET ratio change after every single min at the 5 μM nitrate concentration and in the absence of nitrate. The FRET ratio was changed in response to the addition of nitrate from 1.2 to 1.8 after that saturation point achieved; as beyond this point, no change in the fluorescence emission intensity was observed (Figure 9). These results reflect that the FLIP-NT nanosensor has the potential for the real-time monitoring of nitrate in living cells.

In Eukaryotes. To see whether the FLIP-NT nanosensor can work in the eukaryotic system, yeast (S. cerevisiae, strain BY4247) cells were transformed with FLIP-NT construct. Imaging of FLIP-NT transformed yeast cells showed the successful expression of the nitrate sensor in the cytosol (Figure 10). The addition of 5 μM nitrate with the FLIP-NT expressing yeast cells decreases the fluorescence emission intensity of donor (CFP) fluorophore and increases the fluorescence emission intensity of an acceptor (YFP) fluorophore. The fluorescence emission ratio, YFP/CFP, was recorded for 600 s and found to be increased from 1.2 at 0 s time to 1.7 at 525 s after the addition of nitrate, and after this point, the saturation phase started, which lasts up to 600 s (Figure 11). This data suggested that the designed FLIP-NT sensor can be used for real-time monitoring of nitrate in the eukaryotic system also.

DISCUSSION

We report the construction of a FRET-based genetically encoded nanosensor that measures the nitrate level in living cells. Molecular docking studies were carried out to perceive a different kinds of interactions between the nitrate ion and amino acid residues of NrtA. Docking of compounds or small molecules into the binding site of a receptor and estimation of binding affinities is a crucial part of the structure-based ligand designing processes.17 In the present study, we used molecular docking analysis to estimate the intermolecular distance between the interacting residues of NrtA-nitrate complexes. The interactions site obtained by molecular docking revealed a unique type of architecture that may contribute to structural reinforcement for the nitrate-binding site and additionally helps to achieve different conformational changes linked with the transport of nitrate.18 Hence, the conformational flexibility within the structure of NrtA protein and sandwiched nitrate...
binding cleft makes it suitable biomacromolecules for FRET-based studies. These conformational changes that occur within the two domains of NrtA protein as a result of nitrate binding help to achieve better FRET efficiency.

In general, FRET-based genetically encoded nanosensor utilizes a popular FRET pair, CFP-YFP as a donor and an acceptor. Earlier instead of fluorophores, dyes have been used for developing such sensors. However, the two most frequently cited limitations of organic dyes for live-cell imaging are the necessitating sophisticated approaches for attaching organic dyes to biomolecules and the challenges of ensuring cell permeability while introducing such dyes in living cells. To address these limitations, genetically encoded FPs have been used as unique cell markers, which also reduces the cell toxicity generally shown by exogenous dyes. Moreover, FRET occurs not only on the conversion of the polypeptide (CFP, YFP, and NrtA) species into aggregates (CFP_NrtA_YFP) but also on the nonradiatively energy transfer from a donor to an acceptor molecule. To obtain a quantitative result, a key condition is a spectral overlap between the donor emission spectrum and acceptor absorption spectrum. The sensor makes the use of a recognition element that has the potential to undergo conformation changes encountering the stimuli of interest.

Different genetically encoded biosensors (maltose, molybdate, sulphate, etc.) have been developed by fusing a bacterial periplasmic-binding protein (PBP) with FRET pairs. This PBP undergoes conformational changes like a Venus flytrap plant upon ligand binding. NrtA from Synechocystis species has the homology with the bacterial PBP and belongs to the class II PBP superfamily. This NrtA is a solute-binding component that displays high-affinity to nitrate ABC transporter that captures nitrate in the periplasm. We have speculated from our study that these conformational changes in NrtA protein are sufficient for the spectral overlap between the donor (CFP) and an acceptor (YFP) molecule for the FRET to occur.

In the ligand titration curve experiment, the change in FRET ratio determines the binding isotherm that correlates with the nitrate concentration in a diverse detection range. Our sensor also displays selectivity and sensitivity toward nitrate compared to other ligands. Moreover, the results of prokaryotic and eukaryotic studies suggested that the binding of NO$_3^-$ with NrtA induces a conformational change, which results in increased fluorescence intensity of YFP and decreased fluorescence intensity of CFP, i.e., the change in the FRET ratio occurs in response to ligand (NO$_3^-$). Similar sensors have been established for other nutrients and ions such as sulfate, phosphate, potassium, etc. Previously, stable isotope techniques and radiotracers have been employed for flux analysis and cellular transport processes, but they lack cellular and subcellular spatio-temporal resolution. FRET-based genetically encoded nanosensors pave the way to dynamic quantification of metabolite with cellular and subcellular resolution in a nondestructive manner. The response of our FLIP-NT nanosensor elucidated that the designed nanosensor has the potential for the quantification of flux or to measure transport across the intracellular membranes that could help the researchers to identify the dynamic of nitrate, underlying processes, and their regulatory switch. These nanosensors can be installed into any cell type via transformation or transfection and can visualize metabolites to subcellular compartments with sufficient spatial and temporal resolution. The genetically encoded nanosensor enables the measurement of steady-state concentrations and also reports the dynamic changes at subcellular resolution. The FLIP-NT sensor can be used as an efficient tool to study the real-time intracellular dynamics of nitrate in prokaryotic and eukaryotic systems in a non-invasive manner.

**CONCLUSIONS**

The NrtA of Synechocystis sp. 6803 was successfully transformed into a nontoxic genetically encoded nitrate sensor, FLIP-NT. The convenience of this genetically encoded sensor is its ease of introduction into any cell type in a nondestructive manner and can be used to scan the nitrate dynamics in different subcellular compartments of live prokaryotic and eukaryotic cells. The emission ratio (donor/acceptor) change in response to nitrate reports the fusion of CFP and YFP at N- and C-termini, respectively, of NrtA and demonstrated the potential of this sensor with an acceptable emission ratio change for nitrate tracing. The developed affinity mutants enable us to visualize a diverse range of physiologically relevant nitrate concentrations, i.e., both an increase and decrease concentrations of cellular NO$_3^-$ can be detected. Our studies on FLIP-NT proved it as a useful tool for the study of NO$_3^-$ level and flux that would help the researchers to unravel regulatory mechanisms and NO$_3^-$ homeostasis in cellular compartments by utilizing this tool in the nitrate assimilation pathway. This tool will further contribute to develop strategies for controlling nitrate pollution and enhancing the nutritional value with the sustenance of high crop productivity to modernize environmental and agricultural applications.

**EXPERIMENTAL SECTION**

**In Silico Molecular Modeling of a Nitrate Sensor.** Autodock Vina was used for molecular docking of the selected ligand in the binding cavity of the receptor as described previously. Atomic coordinates of nitrate binding protein (NrtA) of Synechocystis were retrieved from the Protein Data Bank database (PDB ID: 2G29). The docked NrtA complex was further validated and analyzed using “Receptor–Ligand Interactions” modules of Discover Studio 4.0 and visualized by PyMOL. Structure analysis was performed to see the mode of binding and interaction patterns.

**Design of Protein Constructs.** The strain of cyanobacteria, Synechocystis sp. 6803, was procured from Bhabha Atomic Research Centre (BARC), Mumbai, India, and genomic DNA was extracted by following the protocol of Singh et al. (2011) with slight modification (Figure S1). The reporter element (NrtA) is a periplasmic solute-binding protein isolated from the extracted genomic DNA, which belongs to the ATP-binding cassette (ABC)-type transporter of the class II superfamily. The NrtA protein possesses free N- and C-terminal and nitrate binding pockets, which are flexible and show considerable conformational changes. The NrtA sequence (1341 bp) was obtained from the KEGG database. The signal peptide (initial 36 amino acid residues from N-terminal or 108 bp), one-stop codon, and one penultimate codon were removed from the NrtA sequence, and the remaining 1227 bp was amplified using the standard PCR amplification protocol, with a forward primer (5′-acggtaACCCGGACGGGTACAGGTCACGC-3′) and a reverse primer (5′GGactgtTTTTAATAGACTTAATTTT-CAAGC-3′). The restriction sites of AgeI and SpeI were inserted in forward and reverse primers, respectively. The
amplified 1227 bp sequence of NrtA was inserted in pGEM–T Easy vector (Promega, Madison, Wisconsin, USA), and further used to attached with Cyan FP at N-terminus and Yellow FP at C-terminus by following sequential restriction digestion and ligation protocol.

CFP and YFP were amplified from PDH18 vector (YRC Washington, USA) using PCR amplification. CFP was amplified by the forward primer with the XhoI site (5′-CCGctgagATGAAAGGAGAAGACTTTTC-3′) and the reverse primer with the AgeI site (5′-acgctTGATAGTT-CATCCATGCCATGT-3′), while YFP was amplified by the forward primer with the SpeI site (5′-GAcagtAGAGAG-GAACCTTTTCACTGGAA-3′) and the reverse primer with the HindIII site (5′-CCcaagctTTGGTGATAGTTCCATCCATGCG-CAT-3′). The italicized-on primers represent the flanking regions of their respective enzymes. Finally, CFP_NrtA_YFP construct in the pGEM–T Easy vector was obtained. This chimeric CFP_NrtA_YFP cassette was cleared with XhoI and HindIII enzymes and subcloned into the expression vector “pRSET-B” (Invitrogen, Carlsbad, CA, USA), harboring the 6X-Histidine tag for the purification of recombinant fusion protein. The result obtained was named as “FLIP-NT” (Figure S2). The final clone of nanosensor pRSET-B-CFP_NrtA_YFP was confirmed by gene sequencing (Figure S3) and restriction digestion. The designing strategy for the construction of FLIP-NT nanosensor and energy transfer graphic of the FRET-based sensor is described in Figure 12.

Figure 12. Schematic illustration of the FLIP-NT nanosensor. (A) Designing of the constructed nanosensor. (B) FRET illustration of the developed sensor, showing the mechanism of FRET response observed after the addition of nitrate to the system.

**Expression and Purification of the Chimeric Protein.**

The amplification of the FLIP-NT nanosensor (pRSET-B-CFP_NrtA_YFP) was performed in E. coli DH5α, and the E. coli BL21-CodonPlus (DE3) strain was used for protein production. E. coli BL21-CodonPlus (DE3) cells carrying the FLIP-NT nanosensor were grown aerobically at 20 °C in the Luria–Bertani medium (augmented with 100 μg/mL ampicillin and 20 μg/mL chloramphenicol) and induced with 0.8 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) once the culture reached an OD600 = 0.5–0.6. After the induction of IPTG, the cells were grown aerobically for next 18 h at 20 °C in the dark to avoid photobleaching of fluorophores. Thereafter, the culture was pelleted down by centrifugation at 3320×g for 20 min at 4 °C. The pellet was resuspended in 20 mM Tris-Cl pH 8.0 buffer for cell lysis using ultrasonication by following the reported method.35 The insoluble cellular debris was cleared by centrifugation (3320×g for 20 min), and the cleared supernatant was subjected to Ni-NTA chromatography (Qiagen, Germany). Subsequently, bound protein was eluted by elution buffer (20 mM Tris-Cl and 250 mM imidazole, pH 8.0). The eluted sensor protein was stored at 4 °C until further use.

**Characterization of the Recombinant Nanosensor Protein.**

The purified nanosensor protein was subjected to spectral analysis in the absence (control) and presence of 1 μM nitrate. Excitation was given at 435 nm, and emission was recorded from 450 to 600 nm.

Purified sensor protein was resuspended in different buffers ranging from pH 5.0 to 9.0. Buffers used in this study are Tris-Cl, phosphate buffer saline (PBS), and 3-(N-morpholino) propionic acid sulfonic acid (MOPS). The purified sensor protein was diluted 20 times by the respective buffers, and the change in FRET ratio 535 nm/485 nm concerning buffers and pH was analyzed in a monochromator microplate reader (Synergy H1, Biotek, Winooski, VE, USA). To determine the ligand-binding specificity of the FLIP-NT nanosensor protein, the interaction of purified protein was studied with nitrate and other related metabolites, named sulphate, phosphate, chlorate, and bicarbonate with 0, 1, and 5 μM concentrations each.

Experiments were carried out in a 96-well plate in a microplate reader using the excitation filter/slit 430 nm/20 nm, and emission filters/slits for CFP and YFP were 485 nm/20 nm and 535 nm/25 nm, respectively. In each well, 180 μL of the diluted sensor protein and 20 μL of each ligand was added.

To analyze the binding affinity (Kd) of the FLIP-NT sensor protein, the ligand titration curve experiment was carried out in a microplate reader (Biotek, USA) as described previously.36 This affinity assay was performed in a 96-well plate. In each well, a mixture of 180 μL of FLIP-NT sensor protein and 20 μL of nitrate solution with varying concentration ranges (nM to mM) were used. For the estimation of binding affinity (K_d), the ligand titration curve was obtained by plotting the graph of YFP/CFP ratio versus nitrate concentrations and fitted in a simple binding isotherm equation

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S = (r - R_{min})/(R_{max} - R_{min}) = [L]/(K_d + [L])
\]

where S is the representing saturation of binding site, r the FRET ratio, R_{min} is the minimum ratio in the absence of nitrate, R_{max} is the maximum ratio at saturation with nitrate, L is the nitrate concentration, and K_d indicates the dissociation constant. The sensor protein was diluted in MOPS buffer (20 mM) at pH 7.2. The FRET ratio was determined by the acceptor emission intensity at 535 nm divided by the donor emission intensity at 485 nm. All analyses were achieved in triplicates.

**Site-Directed Mutagenesis.**

To expand the dynamic range of the FLIP-NT nanosensor, we measured nitrate levels at physiologically diverse scales, and further to enhance the nitrate detection range, the affinity mutants FLIP-NT-32, FLIP-NT-56, and FLIP-NT-1 were created using site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA). Expression, purification, and affinity assays of mutant nanosensor chimeric proteins were carried out as described in previous sections.

**Real-Time Quantification of Nitrate in Live Cells. In Prokaryotic Model.**

The CFP_NrtA_YFP chimeric construct was transformed in E. coli BL21-CodonPlus (DE3) strain. A single colony was picked and grown on the Luria–Bertani medium for three days at 20 °C augmented with 100 μg/mL ampicillin and 20 μg/mL chloramphenicol until the optical density reaches 0.6, and recombinant nanosensor protein was
expressed at 20 °C in the dark for 18 h by inducing it with 0.8 mM IPTG. The culture was then stored at 4 °C for proper protein folding. The expressed bacterial culture was resuspended in 20 mM MOPS buffer (pH 7.2) for further analysis. The monitoring of the emission ratio (YFP/CFP) was carried out in a microplate reader with a 96-well plate. Each well containing 180 μL of resuspended cells of the FLIP-NT sensor and 20 μL solution of 5 μM nitrate or without nitrate (control), and emission ratio was recorded for 13 min. The excitation filter was set at 420/50 nm, while emission filters for CFP and YFP were set at 485/20 and 535/25 nm, respectively. The bacterial culture was also used for confocal microscopy to get an image of the bacterial cells expressing the FLIP-NT nanosensor.

In Eukaryotic Model. Baker’s yeast S. cerevisiae/URA3 strain BY4247 was used as a unicellular eukaryotic system to validate the efficacy of our developed nanosensor. Gateway cloning technology (Invitrogen, USA) was used in yeast allowing high-speed, reliable, and accurate site-specific recombination. CFP_NrtA_YFP construct from pRSET-B-CFP_NrtA_YFP was shuttled to an entry vector (pDONR222) by following BP-mediated recombination reaction to yield the pDONR222-CFP_NrtA_YFP construct. Subsequently, the construct was shuttled to a destination vector pYES-DEST52 generating the pYES-DEST52-CFP_NrtA_YFP expression clone for LR-mediated reaction. This construct was transformed into the yeast strain BY4247, and growth was obtained at 30 °C by aeration and nutrient availability by proper shaking on an incubator shaker. Plating was done on yeast synthetic dropout medium to remove any plasmids or auxotrophic markers present in yeast. YFP and CFP were set at 485/20 and 535/25 nm, respectively.

Sensor and 20 °C, and emission ratio was recorded for 13 min. The excitation filter was set at 420/50 nm, while emission filters for CFP and YFP were set at 485/20 and 535/25 nm, respectively. The bacterial culture was also used for confocal microscopy to get an image of the bacterial cells expressing the FLIP-NT nanosensor.

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## ASSOCIATED CONTENT

1. Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c04868.

Gel image of extracted genomic DNA of Synochocystis sp. PCC 6803 at 0.8% (w/v) agarose gel (Figure S1); nanosensor construct map in pRSET-B expression vector named as FLIP-NT (Figure S2); and sequencing result of FLIP-NT sensor, full length sequence of CFP_NrtA_YFP construct (Figure S3) (PDF)

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## ABBREVIATIONS

| Abbreviation | Description |
|--------------|-------------|
| FP | fluorescent protein |
| CFP | cyan fluorescent protein |
| YFP | yellow fluorescent protein |
| FRET | fluorescent resonance energy transfer |
| FLIP-NT | fluorescent indicator protein for nitrate |
| PBS | phosphate buffer saline |
| TBS | tris buffer saline |
| MOPS | 3-(N-morpholino) propanesulfonic acid |

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