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A genetically encoded fluorescent biosensor for detecting nitroreductase activity in living cancer cells

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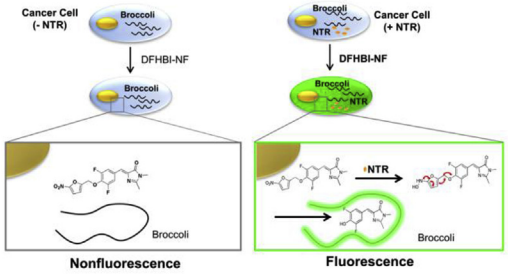
HIGHLIGHTS

- First example of a dual activated fluorescent probe based on RNA mimics of GFP for the detection of nitroreductase.
- Cell imaging nitroreductase only in RNA aptamer expressing cells.
- The universal strategy of quenching fluorescence of the RNA/DFHBI complex by alkylation of the hydroxyl group in the DFHBI.

GRAPHICAL ABSTRACT



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ABSTRACT

Genetically encoded fluorescent biosensors are particularly promising sensors to examine biochemical processes in a complex cellular context. RNA mimics of GFP are RNA-fluorophore complexes that emit green fluorescence comparable in brightness to fluorescent proteins. The fluorophore is non-fluorescent until it binds with specific RNA. We designed and developed a dual activated fluorescent probe based on RNA mimics of GFP for the detection of an intracellular nitroreductase. Our probe only fluoresces in the presence of both the specific RNA and nitroreductase. Since we used RNA to tag cells, our probe only fluoresces when nitroreductase exists in such cells. Our detection system should benefit the study of cell biomarkers in heterogeneous cell populations. More importantly, the strategy of quenching the RNA/DFHBI complex by alkylation of the hydroxyl group in DFHBI opens new possibilities for developing various genetically encoded fluorescent biosensors based on RNA/DFHBI complexes.

1. Introduction

Genetically encoded fluorescent biosensors are particularly promising sensors for researchers to examine biochemical processes in a complex cellular context. These biosensors can be introduced easily into cells, tissues and organisms, and allow long-term imaging for days or longer [1–3]. Genetically encoded green fluorescent protein (FP) sensors can localize at defined regions in a cell by fusing GFP to a signal sequence or a protein that locates to a specific region [4]. A large number of FP-based biosensors have been applied to a variety of biology applications in living cells. The detection mechanisms of FP-based biosensors are divided into three categories: (i) fluorescence resonance energy transfer (FRET) between FP pairs; (ii) alteration of the optical properties of FPs; and (iii) FP splitting. In a FRET-based FP biosensor, analytes modulate the distance, the orientation, or both between the two fluorophores

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[4,5]. Cameleons are one of the oldest families of FRET-based Ca^{2+} sensors, which change color upon binding Ca^{2+} [6]. Alteration of the optical properties of a single fluorescent protein typically by modulation of the protonation state of the chromophore upon ligand-binding results in a spectral shift that is measured, such as pH [7] and halide [8] detection. The split fluorescent protein system involves the use of two tags that template the formation of the green fluorescent protein (GFP) by recruiting each half of the split GFP [9]. The split GFP system has been used previously for protein quantification [10] and visualization of protein subcellular localization [11].

Nitroreductase (NTR) is a flavoenzyme that catalyzes the reductive degradation of a broad range of nitroaromatic compounds to their corresponding amines with β-nicotinamide adenine dinucleotide (NADH) or β-nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor [12,13]. Moreover, reduced oxygen levels in cancer cells can increase NTR activity [14–18]. Thus, detection of NTR activity for biological studies is of significant interest. Substrates of NTR not exist in the structures of natural amino acids. It is impossible to develop FP-based genetically encoded fluorescent biosensor for NTR.

To develop genetically encoded fluorescent biosensor with unnatural substrates, metabolic incorporation of unnatural amino acids is necessary [19–21]. For example, Schultz and co-workers developed a genetically encoded EGFP-K85AcK to detect sirtuin activity [22]. They mutated the lysyl residue of EGFP to N-Ɛ-acetyl-L-lysine (AcK) by co-expressing orthogonal amber suppressor pyrrolysyl-tRNA synthetase mutant (AcKRS)/$tRNA^{Pyl}$ pairs specific for AcK. Deacetylation of the lysyl residue of EGFP by sirtuin restores fluorescence. The metabolic incorporation of unAAs into FPs greatly extends the range of analytes, including H_2S [23,24], ROS [25–29], Cu^{2+} [30], pH [31] and NADH [32,33]. Co-expressing tRNA synthetase mutant increased the complexity of the detection system. To our best knowledge, the genetically encoded fluorescent biosensor for NTR has not been reported yet. Therefore, it would be very useful to develop simple and straightforward approaches to construct genetically encoded fluorescent biosensors for NTR activity detection.

We hypothesized that an alternative approach for preparing a genetically encoded fluorescent biosensor would involve the use of nucleic acid mimics of GFP. Here, an RNA aptamer that binds and induces the fluorescence of fluorophores would resemble that of GFP [34,35]. The fluorophore, 4-hydroxybenzylidene-imidazolinone (HBI), is positioned in the center of GFP and is nonfluorescent in solution [36,37], but is highly fluorescent within the folded GFP. A series of fluorescent nucleic acid aptamer complexes have been obtained, such as Spinach/Spinach2 [38–41], Broccoli [42], Mango [43], Corn [44], and RNA G4 [45]. In 2014, Jaffrey and co-workers described a Broccoli RNA aptamer that binds to 3, 5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), an analogue of the HBI in GFP, leading to its fluorescence [42]. The RNA aptamer Broccoli has a lower dependence on the concentration of magnesium and does not require a scaffold to promote its folding.

Careful examination of the DFHBI molecule revealed that alkylation of the hydroxyl group in the DFHBI should quench its fluorescence [46]. Similar strategies had been used widely in small organic fluorescent probe designs [47–49]. When caged DFHBI reacts with the target molecule, such as a small molecule or enzyme, to release the hydroxyl group, the fluorescence of the RNA/DFHBI complex should be restored. For detection of enzyme activity our design has the following advantages: 1) the caged hydroxyl group in the DFHBI can directly react with the analyte, which avoids possible steric hindrance of the response site located on the protein and the slower diffusion of the protein limiting the reaction kinetics and 2) the caged DFHBI molecule does not need to combine with RNA. DFHBI is regenerated and combines with an RNA aptamer following its reaction with the analyte. Thus, substrate modification does not need to be considered for RNA aptamer binding, which greatly simplifies the design of the substrate.

Herein, we designed and developed a dual activated fluorescent probe based on RNA mimics of GFP for the detection of NTR. We used DFHBI and 2-bromomethyl-5-nitro-furan to synthesize 5-[3,5-difluoro-4-(5-nitro-furan-2-ylmethoxy)-benzylidene]-2,3-dimethyl-3,5-dihydro-imidazol-4-one (DFHBI-NF), which blocks phenolate formation using a protecting group (Scheme 1a) [50]. Our probe can only fluoresce in the presence of both the Broccoli RNA aptamer and NTR (Scheme 1b). Since we used the RNA aptamer to tag cells, our probe only fluoresces when NTR is present in such cells.

2. Methods

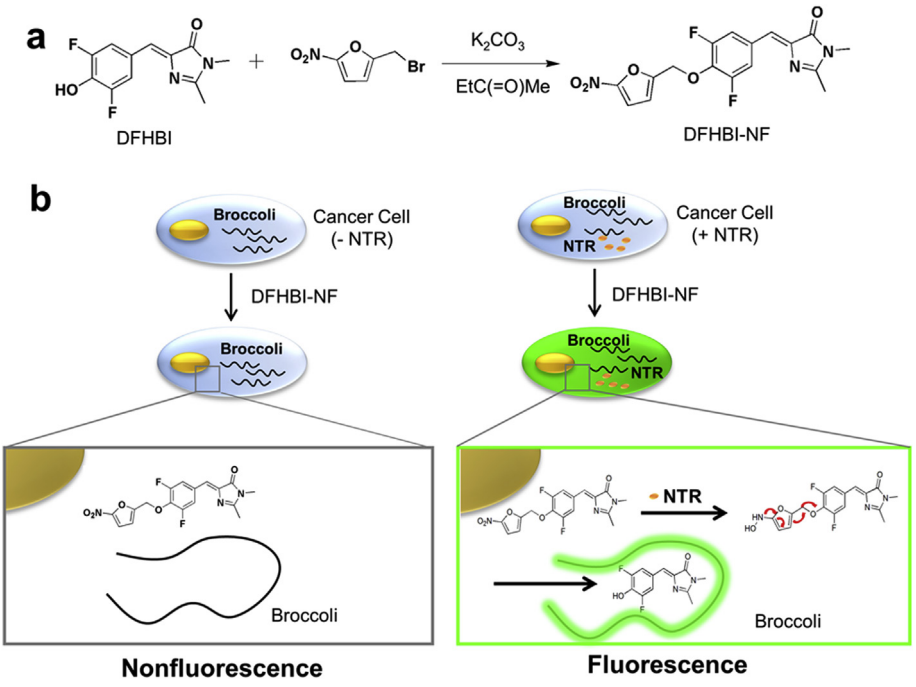
2.1. Chemicals and materials

NTR and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich (Shanghai, China). N-Acetylglycine, 40% CH_3NH_2, and p-nitrobenzoic acid were purchased from Aladdin Industrial Inc. (Beijing, China). 3,5-Difluoro-4-hydroxybenzaldehyde and 2-(bromomethyl)-5-nitrofuran were purchased from J&K Chemical (Beijing, China). Fetal bovine serum (FBS), Lipofectamine® 3000 was purchased from Thermo Fisher Scientific Life Inc. (Shanghai, China). The SanPrep Column Endotoxin-Free Plasmid Mini-Preps kit, Total RNA Extractor (Trizol), and sucrose were purchased from Sangon Biotech Co., Ltd (Shanghai, China). Purified water was taken from a Millipore Simplicity 185 purification unit and was used to rinse and prepare all aqueous solutions. All reagents were obtained commercially and used without further purification. 5-(3,5-Difluoro-4-hydroxy-benzylidene)-2,3-dimethyl-3,5-dihydro-imidazol-4-one (DFHBI) was synthesized as described previously [35]. ^{1}H- and ^{13}C NMR spectroscopy were performed on a Bruker Ascend 600 NMR spectrometer. Electrospray ionization mass spectra (ESI-MS) were collected on a Bruker Maxis ESI-Q-TOF instrument. Absorbance spectra were recorded on a Purkinje General TU-1950 UV–Vis spectrophotometer. Fluorescence emission and excitation spectra were measured using a Hitachi F-7000 spectrofluorometer.

2.2. Cells and plasmids

HeLa cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. E. coli. BL21 competent cells were purchased from Sangon Biotech (Shanghai) Co., Ltd. Plasmid pET28a was a gift from Dr. Yi Qiang (Shaanxi Normal University, Xi'an, Shaanxi, China). Plasmids pET28c-F30-2xdBroccoli (Addgene plasmid #66843) and pAV5S–F30-2xdBroccoli (Addgene plasmid #66845) were a gift from Prof. Samie Jaffrey (Weil Cornell Medicine College, Cornell University, New York, NY, USA) [42].

Synthesis of DFHBI-NF. 5-[3,5-Difluoro-4-(5-nitro-furan-2-ylmethoxy)-benzylidene]-2,3-dimethyl-3,5-dihydro-imidazol-4-one (DFHBI-NF) was synthesized according to a previous report [51]. DFHBI (200 mg, 0.79 mmol), 2-bromomethyl-5-nitro-furan (160 mg, 0.79 mmol) and 126 mg potassium carbonate were refluxed with 5 mL butanone overnight. The reaction mixture was cooled to room temperature, mixed with 10 mL water and stirred for 2 h. The organic layer was separated, dried with anhydrous sodium sulfate and the solvent was removed under reduced pressure. The solid was purified by recrystallization from ethyl acetate to afford the probe as a dark brown solid 200 mg (66.7% yield). ^{1}H NMR (600 MHz, $CDCl_3$), δ 7.74 (d, $J = 9.6$ Hz, 2 H), 7.25 (d, $J = 2.4$ Hz,



Scheme 1. (a) Synthesis of DFHBI-NF. (b) Proposed response mechanism of DFHBI-NF to nitroreductase (NTR) and the fluorescence response of DFHBI-NF to Broccoli.

1 H), 6.86 (s, 1 H), 6.65 (d, $J = 3.6$ Hz, 1 H), 5.22 (s, 2 H), 3.17 (s, 3 H), 2.38 (s, 3 H). ^{13}C NMR (600 MHz, $CDCl_3$), δ 170.3, 164.1, 156.2, 154.5, 152.9, 139.8, 135.2, 130.6, 123.5, 115.7, 115.6, 113.2,111.9, 67.1, 26.6, 15.7. ESI$^+$-HRMS: calcd $[M+Na]^+$ m/z for $[C_{17}H_{13}F_2N_3O_5+Na]^+$, 400.0715; found, 400.0711. (Figs. S1–3).

UV–Vis absorption spectra for nitroreductase detection DFHBI-NF (10 μM) was incubated with NADPH (500 μM) and NTR (10 μg/mL) in 50 mM PBS buffer (pH 7.4). The mixture was incubated for 30 min at 37 °C. UV–Vis absorbance spectra were measured using a Purkinje General TU-1950 UV–Vis spectrophotometer.

Fluorescence spectra for nitroreductase detection. DFHBI-NF (10 μM) was incubated with NADPH (500 μM), NTR (10 μg/mL) and Broccoli in 50 mM PBS buffer (pH 7.4). The mixture was incubated for 30 min at 37 °C Fluorescence spectra were recorded on a Hitachi F-7000 spectrofluorometer using an excitation wavelength of 447 nm. The excitation and emission slits are 10 nm and 10 nm, respectively.

Expression of Broccoli in *E. coli*. We transform *E. coli.* BL21 cells with 40 ng of the pET28c-F30-2xdBroccoli plasmid. Transformed cells were incubated overnight at 37 °C and single colonies were picked for inoculation in Luria Broth containing kanamycin (LB-Kan).

Preparation of *E. coli* cells for imaging. Cells were incubated in LB-Kan for 12–16 h at 37 °C were in shaking (150 rpm). At an OD_{600} of 0.4, IPTG was added to the culture to give a final concentration of 1 mM and shaking (250 rpm) was continued at 37 °C. Cells were harvested by centrifugation and resuspended in 1 mL PBS buffer (pH 7.4). A 200-μL aliquot of the resuspended culture was diluted to 1 mL and prepared for subsequent experiments.

Live cell imaging in *E. coli*. Cells containing the pET-28a plasmid were used as negative control. The above resuspended culture was incubated with 500 μM NADPH, 500 μM p-nitrobenzoic acid and 20 μM DFHBI-NF at 37 °C. Cells containing the pET28c-F30-2xdBroccoli plasmid were selected as the blank control. The above resuspended culture was incubated with 500 μM NADPH, 500 μM p-nitrobenzoic acid and 20 μM DFHBI-NF at 37 °C for 30 min. Cells containing the pET28c-F30-2xdBroccoli plasmid were selected as positive control. The above resuspended culture was incubated with 500 μM NADPH, 500 μM p-nitrobenzoic acid, 20 μM DFHBI-NF and 10 μg/mL NTR at 37 °C for 30 min. All cell images were obtained using an Olympus FV-1200 confocal laser-scanning microscope in the green channel ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 550 \pm 50$ nm).

Flow cytometry analysis of DFHBI-NF in *E. coli*. Cells were treated as described above. All cells were acquired using a BD FACSCalibur flow cytometer.

Expression of Broccoli in HeLa cells. HeLa cells were cultured in DMEM supplemented with 10% FBS at 37 °C in a humidified incubator containing 5% CO_2. The pAV5S–F30-2xdBroccoli plasmid was used for the expression of Broccoli in mammalian cells. Glass-bottom dishes (12-well) were coated with 500-μL poly-L-lysine (PLL) overnight. The dishes were washed once with PBS buffer and HeLa cells were plated at 1×10^5 cells/mL. The cells were cultured in DMEM supplemented with 10% FBS at 37 °C in a humidified incubator containing 5% CO_2. HeLa cells were transfected with 1 μg pAV5S–F30-2xdBroccoli plasmid and the next day with Lipofectamine® 3000. Imaging experiments were performed 48–72 h after transfection.

Live cell imaging of HeLa cells. HeLa cells washed with PBS buffer (pH 7.4) were added to 1 mL PBS buffer containing 300 mM sucrose (sucrose can induce stress granule formation), 500 μM p-nitrobenzoic acid and 20 μM DFHBI-NF (negative control). The culture was incubated for 30 min under normoxia. HeLa cells were washed with PBS buffer (pH 7.4) and added to 1 mL PBS buffer containing 300 mM sucrose and 20 μM DFHBI-NF (blank control). The culture was incubated for 30 min under normoxia. For the positive control, HeLa cells were incubated for 24 h under hypoxia via MGC AnaeroPack® System. The HeLa cells were then washed with PBS buffer (pH 7.4), and 1 mL PBS buffer containing 300 mM sucrose and 20 μM DFHBI-NF was added. The culture was incubated for 30 min. All cell images were obtained using an Olympus FV-1200 confocal laser scanning microscopy in the green channel ($\lambda_{ex} = 488$ nm, $\lambda_{em} = 550 \pm 50$ nm).

3. Results and discussion

We developed a genetically encoded fluorescent biosensor to detect NTR. Our probe is hydrolyzed by NTR to produce fluorescence when bound to Broccoli. The absorption and fluorescence spectra of DFHBI-NF toward NTR were initially measured. In the UV–Vis absorption spectrum (Fig. 1a), the DFHBI-NF showed an absorption peak at 350 nm. A new absorption peak of DFHBI-NF appeared around 420 nm after reacting with NTR in the presence of NADPH, which is the maximum absorption peak of DFHBI [35]. As shown in Fig. 1b, the emission of DFHBI-NF was essentially nonfluorescent because phenolate formation was blocked using a protecting group. The fluorescence emission was enhanced at 522 nm when NTR and NADPH were added to the DFHBI-NF solution. We also tested the fluorescence of Broccoli/DFHBI-NF in the solution of biological relevant ions mixture and amino acids mixture to investigate the effects of coexisting substances in biosystems (Fig. S4). Results showed fluorescence of the RNA/DFHBI-NF system have slightly decreased. And the signal/noise ratio between with or without NTR still are sufficient for cell imaging applications. The results showed that DFHBI-NF serves as a probe to detect NTR.

Two types of E coli cells were then selected to determine if DFHBI-NF could be hydrolyzed by NTR and bind to Broccoli to exhibit a range of fluorescence properties. p-Nitrobenzoic acid was used as the competitive inhibitor of NTR to consume endogenous intracellular NTR [52]. As shown in Fig. 2, DFHBI-NF is nonfluorescent in the absence of Broccoli and NTR. Moreover, incubating cells that contained Broccoli and DFHBI-NF but no added NTR also showed no fluorescence. Bright fluorescent was only observed when Broccoli and NTR were incubated together in the cells. As predicted, our probe only fluoresces in the presence of both the Broccoli RNA aptamer and NTR. We next used flow cytometry to verify the above results. Experimental conditions were the same as above. As shown in Fig. 3c, the FACS histogram shows that more than 60% of the cells were fluorescent when Broccoli and NTR were present. The cells were almost nonfluorescent when Broccoli was absent or both Broccoli and NTR were absent (Fig. 3a and b). Fig. 3d shows the mean fluorescence intensity histogram of (a), (b) and (c). We observed that fluorescence was enhanced significantly when both Broccoli and NTR were present. The results indicated that DFHBI-NF could be hydrolyzed by NTR and bind to Broccoli to exhibit fluorescence.

With the above results in mind, we were confident that our probe would be effective for NTR imaging in living cancer cells. We selected the HeLa cell line as model cells. HeLa cells were transfected with the pAV5S–F30-2xdBroccoli plasmid with Lipofectamine® 3000 to obtain cells containing Broccoli. The blank sample was treated with p-nitrobenzoic acid. Cells incubated under hypoxia were used as the positive control because a decrease of the oxygen level in cancer cells leads to an increase in the level of NTR. The cells were pretreated with 300 mM sucrose, which induces stress granule formation. As shown in Fig. 4, the untransfected cells did not fluoresce under any situation. The transfected cells treated with p-nitrobenzoic acid were nonfluorescent under normoxia. Transfected cells incubated without p-nitrobenzoic acid produced a faint fluorescence under normoxia, whereas transfected cells showed distinct fluorescence without p-nitrobenzoic acid under hypoxia. According to the results, DFHBI-NF could image NTR in living cancer cells.

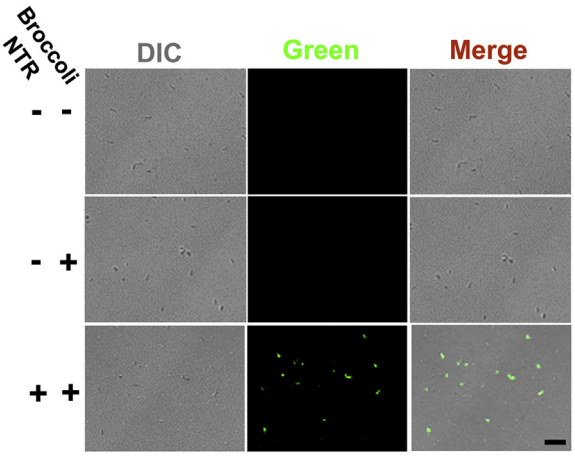


Fig. 2. Confocal fluorescence images of cells in *E. coli*. Cells containing pET-28a plasmid (without Broccoli) were incubated with 500 μM NADPH, 500 μM p-nitrobenzoic acid and 20 μM DFHBI-NF (top). Cells containing the pET28c-F30-2xdBroccoli plasmid were incubated with 500 μM NADPH, 500 μM p-nitrobenzoic acid and 20 μM DFHBI-NF (middle). Cells containing the pET28c-F30-2xdBroccoli plasmid were incubated with 500 μM NADPH, 500 μM p-nitrobenzoic acid, 20 μM DFHBI-NF and 10 μg/mL NTR (bottom). All cells were incubated for 30 min at 37 °C (λ_{ex} = 488 nm, λ_{em} = 500–600 nm). Scale bars denote 10 μm.

4. Conclusion

In conclusion, we designed and synthesized a dual activated fluorescent probe based on RNA mimics of GFP for the detection of NTR. The fluorescent probe was hydrolyzed by NTR and bound to Broccoli to exhibit bright fluorescence. Flow cytometry results verified that fluorescence was enhanced significantly when both

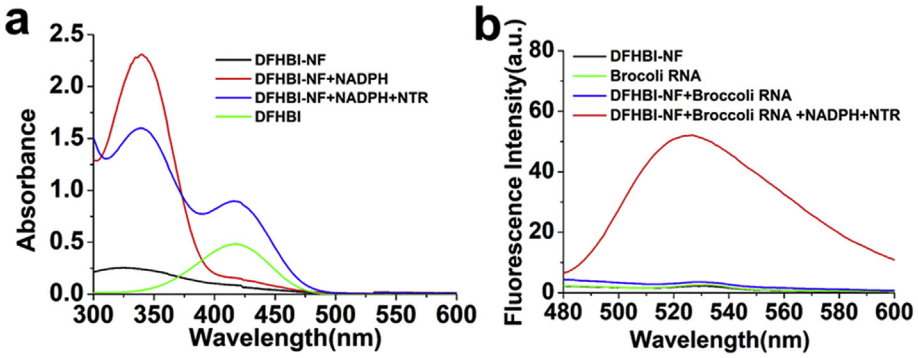


Fig. 1. (a) Absorbance spectra of DFHBI-NF (10 μM) reacting with nitroreductase (10 μg/mL) in the presence of 500 μM NADPH at 37 °C for 30 min. (b) Fluorescence emission (λ_{ex} = 447 nm) spectra of DFHBI-NF (10 μM) reacting with nitroreductase (10 μg/mL) in the presence of 500 μM NADPH and Broccoli at 37 °C for 30 min. The excitation and emission slits are 10 nm and 10 nm, respectively.

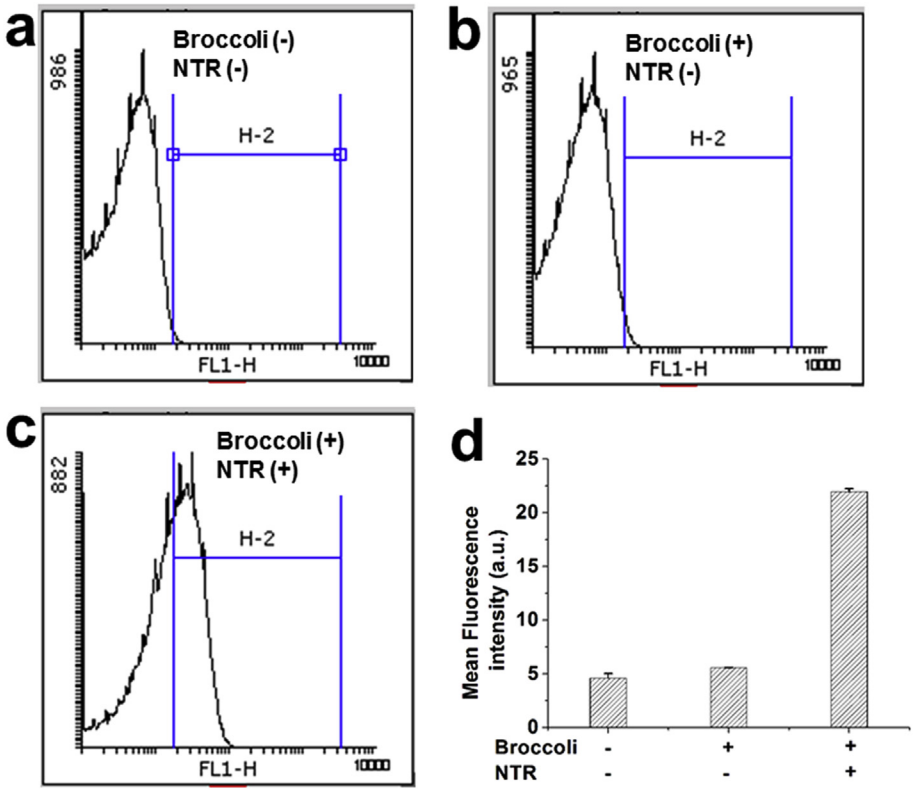


Fig. 3. FACS histogram of cells in *E. coli*. (a) Cells containing the pET-28a plasmid (without Broccoli) were incubated with 500 μM NADPH, 500 μM p-nitrobenzoic acid and 20 μM DFHBI-NF. (b) Cells containing pET28c-F30-2xdBroccoli plasmid were incubated with 500 μM NADPH, 500 μM p-nitrobenzoic acid and 20 μM DFHBI-NF. (c) Cells containing the pET28c-F30-2xdBroccoli plasmid were incubated with 500 μM NADPH, 500 μM p-nitrobenzoic acid, 20 μM DFHBI-NF and 10 μg/mL NTR. (d) Mean fluorescence intensity histogram of (a–c).

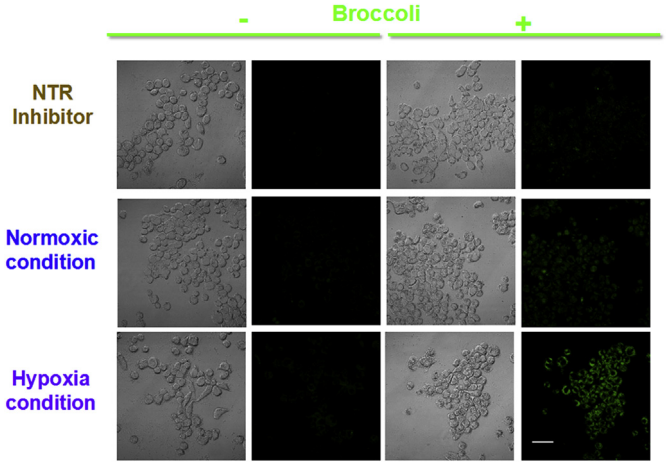


Fig. 4. Confocal fluorescence images of HeLa cells. HeLa cells were incubated with 500 μM p-nitrobenzoic acid and 20 μM DFHBI-NF under normoxia (top). HeLa cells incubated with 20 μM DFHBI-NF under normoxia (middle). HeLa cells incubated for 24 h under hypoxia, HeLa cells were then incubated with 20 μM DFHBI-NF (bottom). All cells were incubated with 300 mM sucrose for 20 min (λ_{ex} = 488 nm, λ_{em} = 500–600 nm). Scale bars denote 50 μm.

Broccoli and NTR were included. We then successfully applied our probe to image NTR in HeLa cells. Thus, our probe only fluoresces in cells using a special RNA tag in the presence of NTR. Our detection system should benefit the study of cell biomarkers in heterogeneous cell populations. More importantly, the strategy of quenching the RNA/DFHBI complex by alkylation of the hydroxyl group in the DFHBI opens new possibilities for developing various genetically encoded fluorescent biosensors based on RNA/DFHBI complexes.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.08.058.

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