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Highlights to

Discriminating changes in intracellular NADH/NAD⁺ levels due to anoxicity and H₂ supply in *R. eutropha* cells using the Frex fluorescence sensor

by S. Wilkening, F.-J. Schmitt, O. Lenz, I. Zebger, M. Horch, and T. Friedrich

- The Frex fluorescence sensor reports [NADH] changes in *Ralstonia eutropha*
- The reduced NADH sensitivity of Frex is suitable for NADH monitoring in bacteria
- Frex signals relate to activity of the soluble hydrogenase (SH) upon H₂ supply
- Frex discriminates NADH changes due to anoxicity from SH activity

Discriminating changes in intracellular NADH/NAD⁺ levels due to anoxicity and H₂ supply in *R. eutropha* cells using the Frex fluorescence sensor

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Keywords:

fluorescence sensor protein; redox sensing; NADH; NAD⁺; Frex; *R. eutropha*; soluble hydrogenase; light-driven bio-hydrogen production

List of abbreviations:

cpFP – circularly permuted fluorescent protein, cpYFP – circularly permuted yellow fluorescent protein, FGN – fructose-glycerol-nitrogen (medium), FN – fructose-nitrogen (medium), Frex – fluorescent Rex, GN – glycerol-nitrogen medium, LB – Luria Bertani (medium), MBH – membrane-bound hydrogenase, NAD – nicotinamide adenine dinucleotide, NAD⁺ – oxidized nicotinamide adenine dinucleotide, NADH – reduced nicotinamide adenine dinucleotide, NADP – nicotinamide adenine dinucleotide phosphate, NADPH – reduced nicotinamide adenine dinucleotide phosphate, NADP⁺ – oxidized nicotinamide adenine dinucleotide phosphate, OD – optical density, RH – regulatory hydrogenase, SH – soluble NAD⁺-reducing hydrogenase, YFP – yellow fluorescent protein

Abstract

The hydrogen-oxidizing “Knallgas” bacterium *Ralstonia eutropha* can thrive in aerobic and anaerobic environments and readily switches between heterotrophic and autotrophic metabolism, making it an attractive host for biotechnological applications including the sustainable H₂-driven production of hydrocarbons. The soluble hydrogenase (SH), one out of four different [NiFe]-hydrogenases in *R. eutropha*, mediates H₂ oxidation even in the presence of O₂, thus providing an ideal model system for biological hydrogen production and utilization. The SH reversibly couples H₂ oxidation with the reduction of NAD⁺ to NADH, thereby enabling the sustainable regeneration of this biotechnologically important nicotinamide cofactor. Thus, understanding the interaction of the SH with the cellular NADH/NAD⁺ pool is of high interest. Here, we applied the fluorescent biosensor Frex to measure changes in cytoplasmic [NADH] in *R. eutropha* cells under different gas supply conditions. The results show that Frex is well-suited to distinguish SH-mediated changes in the cytoplasmic redox status from effects of general anaerobiosis of the respiratory chain. Upon H₂ supply, the Frex reporter reveals a robust fluorescence response and allows for monitoring rapid changes in cellular [NADH]. Compared to the Peredox fluorescence reporter, Frex displays a diminished NADH affinity, which prevents the saturation of the sensor under typical bacterial [NADH] levels. Thus, Frex is a valuable reporter for on-line monitoring of the [NADH]/[NAD⁺] redox state in living cells of *R. eutropha* and other proteobacteria. Based on these results, strategies for a rational optimization of fluorescent NADH sensors are discussed.

Introduction

Ralstonia eutropha is a gram-negative β -proteobacterium endowed with a versatile and adaptive metabolism. The bacterium is able to oxidize organic compounds, preferably organic acids (heterotrophic growth), but is also capable of exploiting CO₂ and molecular hydrogen (H₂) as carbon and energy sources (lithoautotrophic growth), respectively (Cramm, 2009). The ability to metabolize H₂ is mediated by four distinct [NiFe] hydrogenases in *R. eutropha* (Schäfer et al., 2013) that catalyze the (reversible) oxidation of molecular hydrogen in the presence of oxygen (O₂) (Lenz et al., 2015). Two of these O₂-tolerant hydrogenases are directly involved in energy conversion processes of the bacterium. The membrane-bound hydrogenase (MBH) oxidizes molecular hydrogen and channels the released electrons via a membrane-linked cytochrome *b* into the respiratory chain (Kalms et al., 2016). The second hydrogenase is the cytoplasmic soluble hydrogenase (SH), which couples H₂ oxidation to the reduction of oxidized nicotinamide adenine dinucleotide (NAD⁺) to NADH (Horch et al., 2012). NADH, can then be used as a reducing agent for, e.g., CO₂ fixation *via* the Calvin-Benson-Bassham cycle or for ATP generation *via* the respiratory chain (Cramm, 2009). Although biased towards H₂ oxidation, the SH is also capable of proton reduction and H₂ evolution under sufficiently reducing conditions in the cytoplasm (Kuhn et al., 1984). A crucial parameter to estimate the reduction potential of the cytoplasm is the [NADH]/[NAD⁺] ratio, as it (co-)defines the redox states of all the cellular NAD(H)-dependent enzymes and thus their catalytic activity (Lin and Guarente, 2003). Due to its high synthesis rate in living cells, both under aerobic and microaerobic conditions, the bidirectional SH activity affects the [NADH]/[NAD⁺] ratio significantly. It has therefore been postulated that a spectroscopic approach, which combines the measurement of intracellular [NADH]/[NAD⁺] levels with the functional state of the SH in living cells, would be highly beneficial for understanding the catalytic and physiological impact of this enzyme (Horch et al., 2015).

Many approaches have been utilized to measure the [NADH]/[NAD⁺] ratio as well as the individual concentrations of both nicotinamide derivatives in living cells, each with its own advantages and drawbacks. The simplest, yet utterly challenging, approach is to utilize the inherent fluorescence of NADH to estimate cellular concentrations (Rocheleau et al., 2004). However, this method does not allow estimating the ratio between the reduced and oxidized forms, since NAD⁺ itself is not fluorescent. Furthermore, the fluorescence of NADH occurs in a spectral region that severely overlaps with cellular autofluorescence (Patterson et al., 2000). Also, the phosphorylated congener, NADPH, mostly involved in anabolic reactions, cannot be distinguished from NADH by standard fluorescence spectroscopy. Thus, the more demanding

method of fluorescence lifetime analysis is required to discriminate these molecular species (Blacker et al., 2014). However, a quantitative analysis by fluorescence lifetime imaging microscopy (FLIM) is difficult, because complex cellular samples usually show autofluorescence with multiexponential decay kinetics and ambiguous spectral shape.

Other approaches rely on lysis of cells and the investigation of [NADH]/[NAD⁺]-dependent redox couples, e.g., lactate and pyruvate, which are coupled to [NADH] and [NAD⁺] *via* the lactate dehydrogenase reaction (Bilan and Belusov, 2016; Williamson et al., 1967). However, since cells must be disrupted, on-line monitoring of nicotinamide cofactor levels in living cells is impossible with this method.

Recently, genetically engineered fluorescence sensors for the monitoring of [NADH] and [NADH]/[NAD⁺] in mammalian cell lines have been developed (Bilan et al., 2014; Hung et al., 2011; Zhao et al., 2011; Zhao and Yang, 2015). These sensors consist of bacterial NADH-binding proteins (so-called Rex repressor proteins) fused to a circularly permuted fluorescent protein (cpFP) (Baird et al., 1999), whose fluorescence response is highly sensitive towards structural changes. The Rex protein undergoes a significant conformational change upon NADH binding (Sickmier et al., 2005; Wang et al., 2008), which, in turn, modulates the fluorescence response of the cpFP.

Although derived from bacterial Rex repressor proteins, the [NADH] or [NADH]/[NAD⁺] sensors developed to date (Peredox (Hung et al., 2011), Frex/FrexH (Zhao et al., 2011) and SoNar (Zhao et al., 2015)) have been optimized for mammalian cell lines. Importantly, the total cellular NADH/NAD⁺ pools and their ratio may differ substantially in mammalian and bacterial cells (Bennett et al., 2009; Zhao and Yang, 2012; Zhou et al., 2011). Indeed, the Peredox sensor (designed to report the [NADH]/[NAD⁺] ratio) was found to operate close to its saturation level when produced in *R. eutropha* cells grown under aerobic conditions, because its NADH sensitivity is in the lower nanomolar range (Tejwani et al., 2017; Zhao et al., 2011). Therefore, fluorescence sensors with diminished NADH affinity might be advantageous for their application in bacterial cells. Among the various genetically designed fluorescence probes, the Frex sensor has been constructed from the *Bacillus subtilis* Rex protein (genetically optimized to have a lower NADH sensitivity) and the circularly permuted yellow fluorescent protein, cpYFP (Zhao et al., 2011), serving as the chromophore. This sensor was initially established as a tool for determining absolute [NADH].

The goal of this work is to use the Frex sensor for sensing relative changes in NADH levels in *R. eutropha*, taking advantage of its lower NADH sensitivity compared to Peredox (Tejwani et al., 2017), but still bearing in mind the complex interaction of Frex with NADH and NAD⁺

based on our previous work (Wilkening et al., 2017). At NAD^+ concentrations exceeding 100 μM , the oxidized nicotinamide cofactor competes markedly with NADH for the binding site (Gyan et al., 2006) and, thereby, reduces the maximal fluorescence response of the Frex sensor in a concentration-dependent manner (Wilkening et al., 2017). However, since the cellular $[\text{NAD}^+]$ is about two orders of magnitude larger than $[\text{NADH}]$ (Bennett et al., 2009; Zhao and Yang, 2012), even large relative changes in $[\text{NADH}]$ would typically be translated into small absolute changes in $[\text{NAD}^+]$. Consequently, any variation of the Frex fluorescence amplitude should predominantly report just $[\text{NADH}]$ changes, while the corresponding $[\text{NAD}^+]$ variations affect the sensor response only moderately. Indeed, our data show that upon Frex synthesis in *R. eutropha*, its fluorescence signal dynamically reflects changes of cellular $[\text{NADH}]$ during the shift from aerobic to anaerobic conditions and in response to the availability of H_2 , which can be unequivocally assigned to the activity of the SH.

Materials and Methods

Cloning of Frex gene into the pLO13-SH vector. The cDNAs coding for Frex and cpYFP (kind gift from Dr. William Oldham and Prof. Joseph Loscalzo, Harvard Medical School) were cloned into the pLO13-SH vector between the restriction sites of *NcoI* and *HindIII*. The *NcoI* restriction site was introduced by site-directed mutagenesis *via* recombinant PCR. PCR amplification primers were:

Forward Primer Frex: 5'-CGATAAGGAGCCCATGGATAAGG-3'

Forward Primer cpYFP: 5'-GGCTACCATGGCGGATCCG-3'

Reverse Primer: 5'-GCTAGTTATTGCTCAGCGG-3'

The pLO13-SH vector carries a tetracycline resistance gene, and transcription of the subcloned cDNA is under the control of the endogenous promoter for the soluble hydrogenase (SH).

Conjugation of Frex and cpYFP pLO13-SH into the desired R. eutropha strains. *E. coli* S17-1 cells were transformed with the pLO13-SH plasmid vector carrying the cDNA of the fluorescent sensor protein and grown overnight in 10 mL of LB medium containing 10 $\mu\text{g}/\text{mL}$ tetracycline. Recipient *R. eutropha* strains HF798 (SH^+ , MBH^- , regulatory hydrogenase $^-$ (RH^-)) and HF500 (SH^- , MBH^- , RH^-) (Horch et al., 2010; Kleihues et al., 2000) were also cultivated overnight in 10 mL LB medium. Cells were harvested and washed with 5 mL of

sterile H16 buffer (24 mM Na₂HPO₄·7H₂O, 11 mM KH₂PO₄) and resuspended in 1 mL of H16 buffer. 200 µL of donor and recipient strains were given onto a LB agar plate and mixed by spot mating. After 6 h of incubation at 37 °C, the cells were scraped off the plate with a sterile glass pipette, washed in 5 mL of sterile H16 buffer, and resuspended in 1 mL of sterile H16 buffer. 100 µL of 10⁻⁴-fold diluted samples were streaked onto an FN (composition see below) agar plate containing 10 µg/mL tetracycline and grown for 3 days at 37 °C. Transconjugant colonies appeared and incorporation of the desired plasmid was checked by alkaline lysis (pH 12) of cells expressing Frex.

Growth of *R. eutropha*. *R. eutropha* cells were grown in FN medium (24 mM Na₂HPO₄·7H₂O, 11 mM KH₂PO₄, 37 mM NH₄Cl, 0.81 mM MgSO₄·7H₂O, 68 µM CaCl₂·2H₂O, 11 µM FeCl₃·6H₂O, 1 µM NiCl₂, 0.4 % fructose, pH 7.3) or FGN medium (FN medium with 0.2 % fructose and 0.2 % glycerol) at 30 °C. Cells were grown to an OD₆₀₀ of 7 in the presence of 10 µg/mL tetracycline. Before fluorescence measurements of cells, tetracycline had to be removed from the medium, since the emission spectra of tetracycline and the Frex sensor overlap. Thus, cells were harvested and resuspended to a final OD₆₀₀ of 0.5 in fresh tetracycline-free GN medium (24 mM Na₂HPO₄·7H₂O, 11 mM KH₂PO₄, 37 mM NH₄Cl, 0.81 mM MgSO₄·7H₂O, 68 µM CaCl₂·2H₂O, 11 µM FeCl₃·6H₂O, 1 µM NiCl₂, 0.4 % glycerol, pH 7.3).

Alkaline Lysis. *R. eutropha* cells were grown heterotrophically, harvested, and resuspended to a final OD₆₀₀ of 0.5 in H16 buffer (24 mM Na₂HPO₄·7H₂O, 11 mM KH₂PO₄) at pH 11. Cells were incubated for 15 minutes, leading to lysis of the cells due to the alkaline environment. Suspensions of disrupted cells were subsequently examined by fluorescence spectroscopy.

Gas incubation. 1 mL aliquots of cell suspensions with a final OD₆₀₀ of 0.5 were bubbled for various time periods in a 2 mL quartz cuvette sealed with a rubber septum at a gas flow rate of 0.1 L/min at room temperature. Spectra were recorded immediately afterwards.

Gas mixtures. Cell suspensions with a final OD₆₀₀ of 0.5 were added to a mixture of aerated GN medium and H₂-saturated GN medium in appropriate volume combinations to obtain the desired H₂ percentages. Cuvettes for fluorescence measurements were completely filled by the fluid phase.

Fluorescence measurements. Fluorescence was measured with a Fluoromax-2 spectrofluorometer (Horiba Jobin Yvon, Bensheim, Germany). Slits were set to 4 nm for excitation and 2 nm for emission, while the integration time was 1 s and the increment 1 nm. Frex was excited at 480 nm, and emission spectra were recorded in a suitable range centred around the emission maximum of 515 nm for evaluation of the amplitudes. cpYFP was excited at 480 nm for comparability of the fluorescence response with the derived sensors.

The titration data of the acquired fluorescence intensity F of Frex in lysed cells were analyzed by means of the Hill equation,

$$F = \frac{F_{\max} \cdot [\text{NADH}]^n}{K_D^n + [\text{NADH}]^n} \quad (\text{Eq. 1})$$

in which F_{\max} is the fluorescence in the presence of fluorescence-saturating NADH concentrations, K_D is the microscopic dissociation constant, and n is the cooperativity parameter.

Another approach to determine the dissociation constant K_D for the situation in lysed cells uses a quadratic formula to fit the titration data. In contrast to a classical hyperbolic curve (such as the Hill function in Eq. 1), the underlying model is free from assumptions regarding the concentrations of total and free titrant (receptor) and the binding stoichiometry (Swillens, 1995; Hulme and Trevithick, 2001).

$$\frac{[RL]}{[R_{\text{tot}}]} = \frac{[L_{\text{tot}}] + [R_{\text{tot}}] + K_D}{2 \cdot [R_{\text{tot}}]} - \sqrt{\left(\frac{[L_{\text{tot}}] + [R_{\text{tot}}] + K_D}{2 \cdot [R_{\text{tot}}]}\right)^2 - \frac{[L_{\text{tot}}]}{[R_{\text{tot}}]}} \quad (\text{Eq. 2})$$

In this formula, $[RL]$ is the concentration of the Frex sensor protein with NADH bound (here reflected by the normalized fluorescence amplitude), $[R_{\text{tot}}]$ is the total concentration of equivalent receptor binding sites (in μM), $[L_{\text{tot}}]$ is the total concentration of NADH present (“ligand”), and K_D represents the dissociation constant. Fitting the data using this equation yields two fit parameters, $[R_{\text{tot}}]$ and K_D . If the binding stoichiometry n is known, the total receptor concentration can be calculated from the ratio $[R_{\text{tot}}]/n$.

Data processing was carried out using Origin 2017 software (OriginLab Corp., Northampton, MA, U.S.A.).

Results

The cDNA for the Frex sensor was subcloned into the pLO13-SH vector, as described in materials and methods and previously (Tejwani et al., 2017). The resulting plasmid was introduced into the SH-expressing and SH-deficient *R. eutropha* strains HF798 (SH⁺, MBH⁻, RH⁻) and HF500 (SH⁻, MBH⁻, RH⁻), respectively (Horch et al., 2010; Kleihues et al., 2000). Since the fluorescence of the cpYFP moiety of the sensor increases strongly at alkaline pH (Schwarzländer et al., 2014), expression and functionality of the Frex sensor was confirmed *via* alkaline lysis of cells and subsequent measurement of the Frex fluorescence signal upon this alkaline treatment (Supplementary Figure 1).

We first aimed at inferring changes in cytoplasmic [NADH] by monitoring the fluorescence of Frex to establish a relationship between Frex fluorescence and SH activity by comparing Frex fluorescence in SH-synthesizing and SH-deficient *R. eutropha* strains. Therefore, we sought to elevate Frex fluorescence by treating Frex-synthesizing cells with H₂. To delimit the effect of the H₂-driven NAD⁺ reduction activity of the SH on the cellular NADH pool from the more general effects of anoxic conditions and concomitant overreduction of the cytoplasm, we carried out control experiments with helium (He) treatment.

In a first set of experiments, Frex-containing *R. eutropha* cultures were treated with H₂ gas in a septum-sealed quartz cuvette under constant stirring, until a steady-state fluorescence amplitude was reached (10 minutes). This treatment led to a strong increase in the Frex fluorescence signal by about the same factor for the cells of both *R. eutropha* HF798 and HF500 (Fig. 1A,B).

The robust increase of the Frex signal upon H₂ treatment (Fig. 1A,B) indicates an increase in cytosolic [NADH]. This is in line with the expectation that treatment of *R. eutropha* cells with H₂ removed all O₂ from the samples. Since O₂ serves as the terminal electron acceptor of the respiratory chain (Cramm, 2009), its absence prevents NADH from being re-oxidized by Respiratory complex 1 (NADH:ubiquinone oxidoreductase), and NADH should accumulate in the cytoplasm. Notably, upon H₂ treatment, the fluorescence of the Frex sensor increases more than eightfold in both strains (Fig. 1A,B) indicating that the removal of O₂ and the concomitant blockage of the respiratory chain is mainly responsible for the acute rise in [NADH], irrespective of the presence or absence of the SH. Based on our previous study on the purified Frex protein, an eightfold increase of the Frex fluorescence corresponds to the

maximal extent of sensor signal augmentation at neutral pH. This dynamic range can only be achieved if the sensor response is saturated by NADH, and the free NAD⁺ concentration in the cells is below 100 μM; otherwise NAD⁺ would limit the maximal Frex response to NADH, as shown previously (Wilkening et al., 2017).

To test the hypothesis that O₂ removal is the main factor determining the acutely elevated [NADH] levels independent from the presence of SH, anoxic conditions were also induced by treatment with He gas (Fig. 1A,B). Upon He treatment, an elevated Frex signal of comparable relative amplitude was again observed in both (SH-expressing and SH-deficient) strains, and a rather fast signal decrease to the initial level occurred upon re-aeration (Fig. 1D,F) similar to the time course of the Frex signal observed for SH-deficient HF500 cells upon H₂-treatment (Fig. 1E). The Frex signal increase caused by He treatment reached only 50 % of that of H₂-treated cells. This behaviour might demand further elucidation, but since the same result was obtained for both *R. eutropha* strains (Fig. 1A,B), it is obviously not related to the presence or absence of SH activity. The observed differences might rather hint at the influence of the different physico-chemical properties of the two gases on the complex cellular response that eventually governs the NADH/NAD⁺ levels in an anoxic response. For example, different Henry constants (Sander, 2015), diffusion constants (Engineering ToolBox, 2008) or water/lipid partition coefficients would all influence the availability of a particular gas in cells.

Remarkably, we found no significant difference regarding the H₂-mediated *relative* increase of Frex fluorescence intensity in both strains (note that the overall fluorescence amplitudes in these experiments is dependent on cell density and expression level of the sensor). Thus, the immediate increase of the sensor fluorescence alone does not allow for discriminating effects on cellular [NADH] related to the presence of SH. However, the time courses of the decline of the fluorescence signals during re-aeration of the H₂ exposed cells were strikingly different for the two strains. In SH-deficient cells (HF500), the signal decreased to the initial basal value after about two minutes of aeration (Fig. 1E). In contrast, for the SH-expressing cells of the HF798 strain, the signal stayed elevated for about 30 minutes after re-exposure to atmospheric O₂, and only after this period, the signal dropped sharply over the course of about one minute to the initial value (Fig. 1C). This indicates that in the presence of H₂, continuous activity of SH leads to a steadily elevated [NADH] in strain HF798 (as long as H₂ is available, see below) but not in the SH-deficient strain HF500.

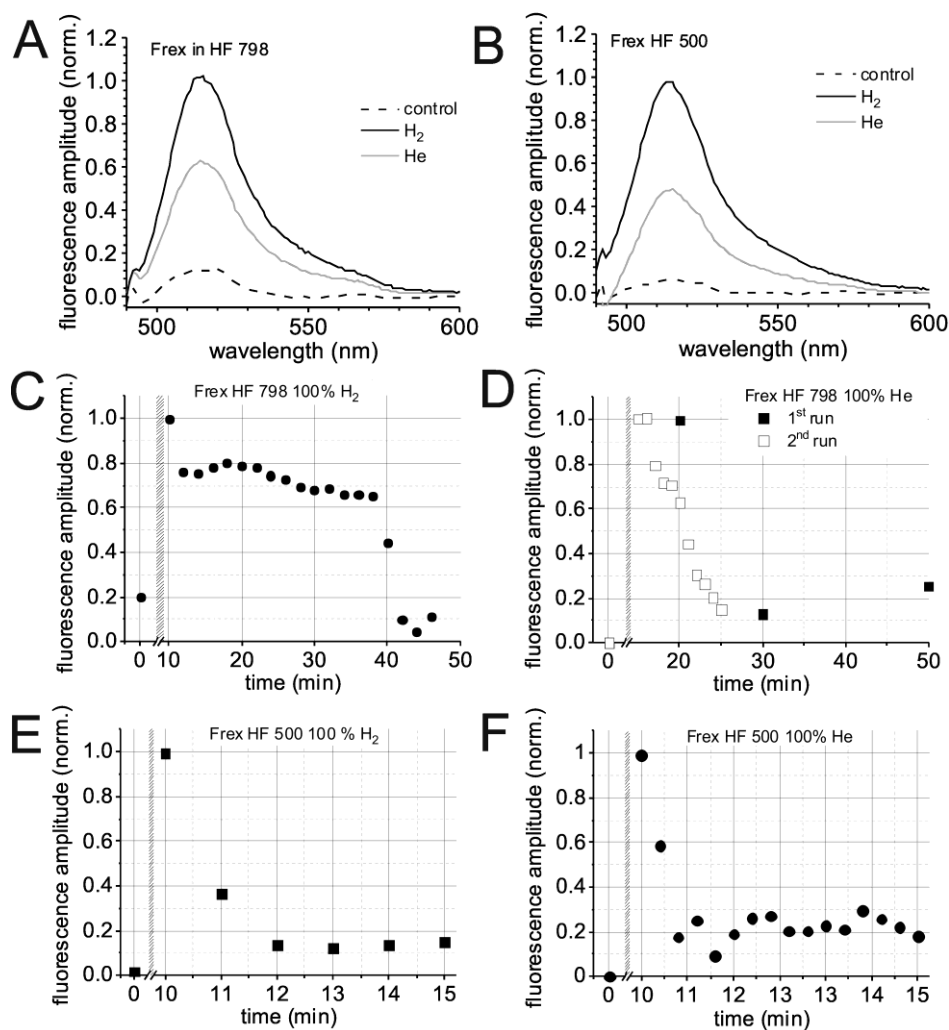


Figure 1: (A,B) Background-corrected and normalized fluorescence spectra of Frex-containing *R. eutropha* cell suspensions of the same OD₆₀₀ after excitation at 480 nm (see Supplementary Figure 2 for the background subtraction procedure). The relative increase in Frex fluorescence is higher for H₂ treatment than for helium treatment in both, the SH-expressing strain HF798 (A) and the SH-deficient strain HF500 (B), indicating that these immediate responses to anoxic conditions occur independent from the presence or absence of the SH. (C,D) Frex fluorescence response in the SH-expressing strain HF798 during and after treatment with H₂ (C) and helium (D). Panel (D) summarizes two experiments conducted on different time scales. (E,F) Frex fluorescence response in the SH-deficient strain HF500 during and after treatment with H₂ (E) and helium (F). Samples in (C-F) were treated in a sealed fluorescence cuvette with H₂ / He gas until the signal was stable, and then the septum was removed (axis break). Data points in (C-F) refer to background-corrected fluorescence levels at 515 nm (excited at 480 nm) normalized to values between 0 and 1, with 0 being the lowest fluorescence amplitude (at the Frex emission peak around 515 nm) and 1 the corresponding maximum amplitude measured immediately after removal of the septum.

At present, it is impossible to derive reliable quantitative information about NADH/NAD⁺

concentrations from the observed sensor responses, since the interaction of NADH and NAD^+ with the Frex sensor is complex, as exemplified by the fact that the dynamic range of the sensor is limited, if NAD^+ concentrations exceed $100 \mu\text{M}$ (Wilkening et al., 2017). Thus, without further knowledge about the actual NAD(H) pool size, it is impossible to discriminate, whether the 50%-reduced response of the sensor in He- versus H_2 -treated cells is, for example, due to a half-maximal NADH concentration ($3.5 \mu\text{M}$) at zero $[\text{NAD}^+]$ or due to the presence of $100 \mu\text{M}$ NADH at about $400 \mu\text{M}$ NAD^+ (see Fig. 1 and Fig. 2 in (Wilkening et al., 2017)). However, the very similar temporal Frex response pattern observed upon He treatment and subsequent re-aeration (Fig. 1D,F) shows that under anoxic conditions and recovery thereof, the cellular response is apparently independent from the presence of the SH. Upon limitation of the terminal electron acceptor O_2 , NADH accumulates, but, as soon as O_2 is available again, the fluorescence signal decreases on a relatively short time scale due to respiratory NADH consumption. In contrast, the constant high-level signal of the sensor, which only occurred in SH-expressing cells after H_2 treatment (Fig. 1C) indicates sustained elevation of $[\text{NADH}]$ due to the activity of the SH. The enzyme should be able to produce NADH as long as sufficient H_2 concentrations are present in the cells during the recovery of atmospheric conditions (see below). This indicates that under the chosen experimental conditions, sufficient H_2 is available for a certain time span (during re-exposure to air), before it is depleted from the solution by diffusion or consumption by the SH.

A caveat for using the Frex sensor in living cells is its pronounced pH-sensitivity, which is inherited from the cpYFP fluorophore (Day and Davidson, 2009; Schwarzländer et al., 2014; Zhao et al., 2011). cpYFP fluorescence can also be used to monitor possible intracellular pH changes. To delineate, whether cytoplasmic pH changes occur during the chosen experimental conditions, gas exchange experiments were carried out with *R. eutropha* cells synthesizing just the cpYFP fluorophore. When cpYFP-containing *R. eutropha* cells, cultivated under the same conditions as the Frex expressing cells used for the experiments shown in Figure 1, were subjected to the same H_2 treatment as described in Figure 1 or to anaerobic conditions by flushing with He gas, the fluorescence amplitude did not change significantly (Supplementary Figure 3). Thus, substantial pH changes influencing the sensor's response can be excluded. Since the cpYFP fluorescence signal of intact cells was rather small, we needed to ascertain appropriate expression of cpYFP. For this purpose, the cells used for the experiments shown in Supplementary Figure 3 were subsequently lysed by resuspension in pH 11 buffer. After this treatment, strongly elevated fluorescence signals were observed, which demonstrates robust cpYFP synthesis in both *R. eutropha* strains. These control experiments support the

notion that the Frex signals observed under the conditions in living cells are not biased by concomitant pH changes in the cytoplasm.

Another set of experiments aimed at a differentiated response of the Frex sensor to different H_2/O_2 ratios. For this purpose, pelleted cells were resuspended in different mixtures of a buffer equilibrated with the oxic atmosphere and another buffer saturated with H_2 . By mixing these two buffers in different ratios and filling the cuvette completely, different H_2 partial pressures could be realized while still having substantial O_2 in the samples. These experiments revealed that the more H_2 is available as SH substrate, which is metabolized to produce NADH, the longer the Frex fluorescence signal remains at an elevated level (Fig. 2A). The duration of elevated fluorescence shows a monotonous increase with increasing H_2 partial pressure, whereas the maximal increase in fluorescence did not depend on this parameter (Fig. 2A). This supports the finding of Fig. 1C that the Frex fluorescence signal stays elevated until all H_2 is consumed by SH (or diffused into the atmosphere) so that the NADH level drops significantly below the saturation level of the sensor.

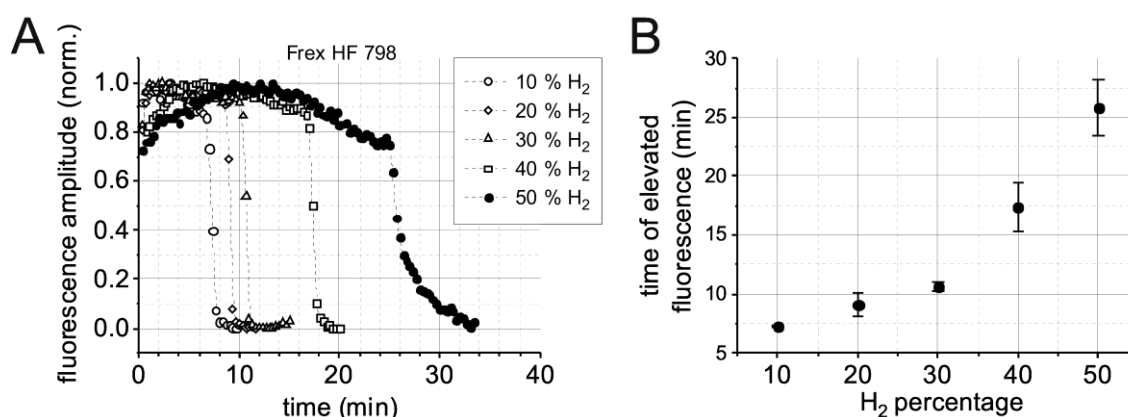


Figure 2: (A) Frex fluorescence in the presence of specified H_2 /air mixtures (H_2 content in % v/v as indicated) in the SH-expressing strain HF798. Excitation was carried out at 480 nm, and fluorescence emission maxima at 515 nm were plotted at various time points. Fluorescence signals were background-corrected and subsequently normalized to values between 0 and 1, with 0 being the lowest fluorescence amplitude (at the Frex emission peak around 515 nm) and 1 the corresponding maximum amplitude measured in the experiment. (B) Influence of the applied H_2 percentage in the gas mixture (partial pressure) on the duration of elevated Frex fluorescence (time for signal decrease to 50% of the saturation value from experiments in panel (A)).

This indicates that even the lowest H_2 concentration applied (10 % of partial pressure corresponds to about $80 \mu M H_2$ as inferred from the Henry constant of H_2 in aqueous solution, see (Sander, 2015)) was sufficient to allow the SH to produce [NADH] exceeding the saturation limit of the Frex sensor. Therefore, rather than the fluorescence intensity, which might be saturated over a wide range of H_2 concentrations, the time duration of the elevated sensor fluorescence serves as a marker for the activity of the SH in living cells (Fig. 2B) in relation to availability of H_2 .

Since the H_2 concentration in the cell suspensions directly relates to the duration of elevated fluorescence, it should also be possible to alter the duration of elevated fluorescence not by changing substrate (H_2) concentration, but by changing the amount of cells (i.e. the cell density) able to consume a given H_2 concentration. This was verified by exposing HF798 cell suspensions of different optical densities to the same H_2 partial pressure (Fig. 3).

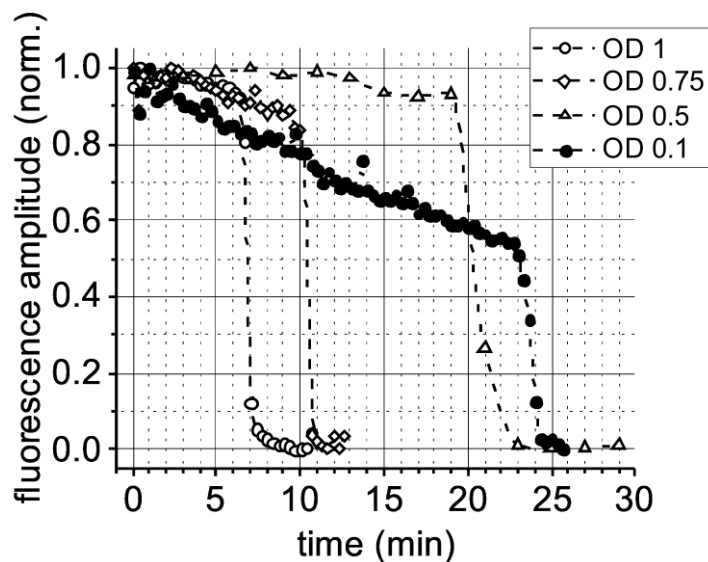


Figure 3: Frex fluorescence signals in the presence of 50 % H_2 in *R. eutropha* HF798 cell suspensions of differing OD. Frex was excited at 480 nm and the fluorescence emission maxima at 515 nm were plotted at various times. The higher the total amount of SH in the samples (due to different cell densities indicated by the OD values), the faster the substrate H_2 is metabolized. Fluorescence signals were background-corrected and subsequently normalized to values between 0 and 1, with 0 being the lowest fluorescence amplitude (at the Frex emission peak around 515 nm) and 1 the corresponding maximum amplitude measured in the experiment immediately after preparation of the samples.

To correlate the observed times of elevated (saturated) Frex sensor fluorescence in experiments on *R. eutropha* cell suspensions in buffers with different H_2 partial pressures

(Fig. 2) or with different cell densities (OD_{600} values, Fig. 3) with the activity of the SH, we performed a hydrogenase activity assay. For this, *R. eutropha* cell suspensions were diluted to a certain OD in an H_2 -saturated buffer containing 1 mM NAD^+ and 0.005 % (w/v) of the surfactant cetyltrimethylammonium bromide (CTAB) to permeabilize the cells (see Supplementary Information). The metabolic activity of the hydrogenase was monitored by the increase of the NADH absorbance (extinction coefficient) at 365 nm over time. From these experiments, the NADH-producing activity of a cell lysate obtained from a suspension of cells of a certain OD could be determined. The H_2 concentration of a 100 % H_2 -saturated aqueous solution is about 800 μ M, as calculated from the corresponding Henry constant of H_2 gas (Sander, 2015), and H_2 concentrations at lower percentages can be determined, accordingly. With these $[H_2]$ values and the metabolic activity stated above, one can calculate the “reaction times” (t_{calc}), which *R. eutropha* cell suspensions of a given OD would need to metabolize a given $[H_2]$, in order to compare these with the experimentally observed times (t_{exp}) of elevated Frex fluorescence from Figure 2B and Figure 3, as listed in Supplementary Tables 1 and 2. Of note, the calculated “reaction times” were in good agreement with the experimentally determined data, the only significant deviation occurred for the experiment with low cell density ($OD_{600} = 0.1$).

To reveal information about the intracellular concentrations of NADH and NAD^+ in *R. eutropha*, we used a calibration technique as described previously for the fluorescence reporter Peredox in lysed cells (Tejwani et al., 2017). First, the Frex-expressing cells were sonicated, which led to a strong decrease of the fluorescence signal because of the dilution of the cellular constituents upon lysis. These lysates were subsequently used for NADH and NAD^+ titration experiments while the corresponding fluorescence emission spectra were recorded (Figure 4).

Figure 4A shows the raw spectra obtained from the crude cell extract incubated with different amounts of NADH. The Frex sensor retained functionality after lysis and shows a dose-dependent response towards NADH. Compared to previous experiments on the purified sensor protein (Wilkening et al., 2017), in which an increase of the Frex fluorescence signal was already observed at 1 μ M NADH, somewhat higher NADH concentrations ($[NADH] \geq 5$

μM) were required to enhance the sensor emission above the background signal. At $[\text{NADH}]$ higher than $500 \mu\text{M}$, the fluorescence emission signal was saturated.

The concomitant NAD^+ titration of the crude cell extract is shown in Figure 4B. First, a constant amount 1 mM NADH was provided to robustly induce sensor emission. Subsequently, increasing NAD^+ concentrations were added. This strategy was chosen because it has been previously shown that NAD^+ reduces the emission of the Frex sensor by competing with NADH for the binding site(s) (Wilkening et al., 2017). Accordingly, we observed decreasing fluorescence signals with increasing $[\text{NAD}^+]$. As already mentioned for the NADH titration experiment, also for the NAD^+ titration a somewhat larger concentration was needed to induce a significant signal decrease compared to the value determined with purified protein (Wilkening et al., 2017). Such deviations, which result in larger apparent K_D values determined from curve fitting, could arise if the concentration of the Frex sensor in the cell lysates would be in the same range as the K_D determined under well-defined conditions on the purified Frex protein (Hulme and Trevithick, 2010; Swillens, 1995). It also seems likely that the debris of cell constituents or membranes non-specifically scavenges NAD(H) to reduce the free NAD(H) concentration.

For quantitative data analysis, the spectra were background-corrected by subtracting the first spectrum after lysis (recorded at zero $[\text{NADH}]$ or $[\text{NAD}^+]$) from the subsequently recorded titration spectra. The spectra recorded in the absence of exogenously added nicotinamide cofactors do not show a significant contribution of Frex-specific emission and resemble the scattering background of the turbid lysates. Peak intensities of the background-corrected spectra at 515 nm were then normalized to values between 0 and 1, plotted against the NADH concentration (Fig. 4C), and fitted by a Hill function (Eq. 1) with the parameters shown in the inset. The calculated K_D value (microscopic dissociation constant) was $(19.6 \pm 1.7) \mu\text{M}$ (with a Hill coefficient 1.7 ± 0.2), which is larger than the K_D value of $3.5 \mu\text{M}$ determined previously for the purified Frex protein (Wilkening et al., 2017). For a better analysis, we used a quadratic formula (Eq. 2) for fitting the data, which is derived from a reaction model that is free from assumptions regarding the concentrations of total and free titrant (receptor) as well as the binding stoichiometry (Hulme and Trevithick, 2010; Swillens, 1995). This model function compensates for the unknown receptor/sensor concentration, while determining the real dissociation constant. The K_D value derived from this fit (Fig. 4D) is about $(5.7 \pm 1.5) \mu\text{M}$, which is in better agreement with the determined K_D from measurements using the purified sensor protein ($K_D = 3.5 \mu\text{M}$, (Wilkening et al., 2017)).

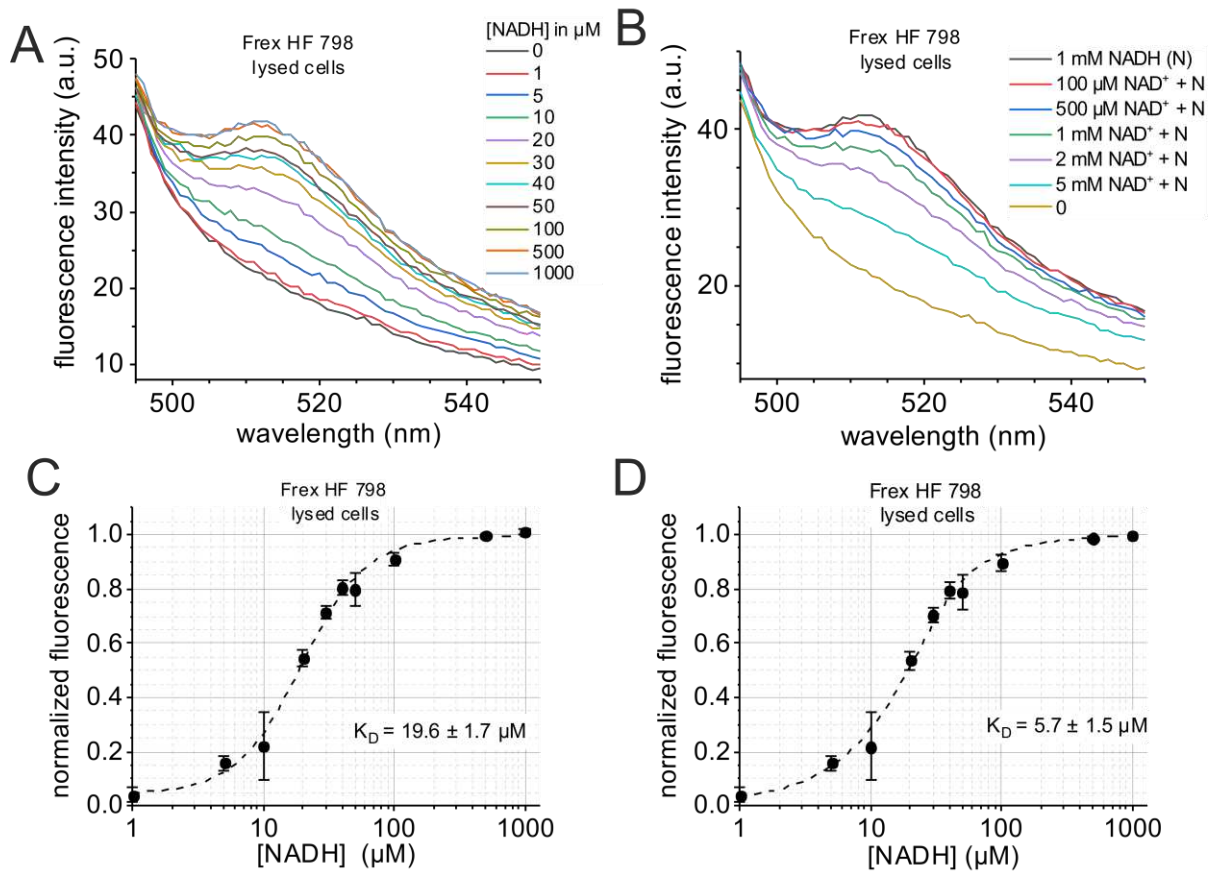


Figure 4: Calibration for the Frex sensor response to NADH and NAD⁺ in lysates of Frex-expressing cells with an OD of 0.1. (A,B) Unprocessed fluorescence emission spectra recorded in *R. eutropha* HF798 cell lysates upon excitation of Frex with 480 nm in the presence of various NADH concentrations (A) or different NAD⁺ concentrations at a fixed [NADH] of 1 mM (B). Fluorescence signals were background-corrected and subsequently normalized to values between 0 and 1, with 0 being the lowest fluorescence amplitude (at the Frex emission peak around 515 nm) and 1 the corresponding maximum amplitude measured in the experiment. (C) Fits of the background-corrected and normalized fluorescence amplitudes from experiments as described in (A) with a Hill function. The resulting fit parameters are given in the inset (data from three experiments). (D) Fit of the data from (C) with a quadratic formula (eq. 2). The inset shows the resulting fit parameters (data from three experiments).

Discussion

In order to determine differences in cytoplasmic NADH levels related to the activity of the soluble hydrogenase (SH), the fluorescence sensor Frex was stably expressed in different *R. eutropha* strains either containing (strain HF798) or devoid of the SH (strain HF500). The

monitoring of dynamic changes in [NADH] is of paramount importance to infer the metabolic status (redox state) of the cells. In theory, the different NADH levels in both cell strains should be attributable to the presence or absence of SH activity, especially if H₂ as substrate for the SH is available. However, control experiments are required to distinguish changes in [NADH] which are related to the activity of the SH from general effects of anaerobic conditions on the respiratory chain.

In measurements with H₂-saturated cell suspensions of strain HF798 (SH⁺), a strong elevation of Frex sensor fluorescence was observed that reached a constant maximum level, which stayed constant for substantial time periods after re-aeration of the cell suspensions, and then dropped sharply to its basal value (Fig. 1C). In comparison, cells devoid of the SH showed a different time pattern of Frex sensor fluorescence. After a short fluorescence emission peak, the signal decreased upon re-oxygenation of the samples rather rapidly within about five minutes or less (Fig. 1E), a pattern that was also observed in both strains, if He treatment was used to induce anoxic conditions. Altogether, this indicates that the expressed SH in strain HF798 metabolized the supplied H₂ to yield NADH as long as sufficient H₂ is available in the system (Schneider and Schlegel, 1976). This activity of the SH keeps the [NADH]/[NAD⁺] ratio sufficiently high in order to stimulate Frex fluorescence over a prolonged period of time. Therefore, the Frex sensor is a convenient tool to monitor SH activity in living cells. Remarkably, initial elevation of the Frex fluorescence signal is apparently independent from the applied H₂ partial pressure, while the duration of the elevated fluorescence under re-aeration is correlated with the previously applied H₂ partial pressure (Fig. 2A) or the cell density (Fig. 3). These findings indicate (i) that the response of the Frex sensor is rapidly driven into saturation during gas treatment due to SH-dependent NADH production – even at low H₂ partial pressures – and (ii) that the duration of the elevated fluorescence signal is indicative of the amount of SH and its substrate (H₂) present during the experiment (Fig. 2B and Fig. 3).

Thus, using the strategies established herein, the Frex [NADH] sensor is capable of determining SH activity in living cells in a variety of settings, since it shows a robust fluorescence response with a large dynamic range, which is dependent on the cellular NADH level. In contrast, the similar Peredox reporter protein with its NADH affinity in the low nanomolar range and its limited dynamic range (Hung et al., 2011; Zhao et al., 2011), showed only minute fluorescence increases under the same experimental conditions (Tejwani et al., 2017). Therefore, the about 100-fold lower NADH affinity of Frex provides a more valuable

tool for NADH detection in cellular settings under which NADH concentrations in the range of hundreds of micromolar are common, as in bacteria, with *R. eutropha* as a biotechnologically relevant example. However, the NADH levels in *R. eutropha* cells, especially under conditions of high SH activity, apparently also exceed the K_D value of the Frex sensor by far, so that the sensor cannot report the full range of cellular NADH concentrations. To adapt to this, further tailoring of the NADH affinity of the Frex sensor would be required. Nonetheless, although the Frex fluorescence response does not differ largely in intensity when exposed to different H_2 partial pressures, it reports variant NADH levels in terms of the time period of the elevated fluorescence signal.

Even though the qualitative response of Frex can be unequivocally assigned to SH activity, it is not possible to quantitatively determine cellular NADH levels due to the antipodal effects of [NADH] and [NAD⁺] on the sensor response (Wilkening et al., 2017). While it was initially reported that the Frex sensor does not interact or alter fluorescence upon binding of the oxidized congener NAD⁺ up to concentrations of 100 μ M (Zhao et al., 2011; Zhao and Yang, 2012), we found later that NAD⁺ concentrations surpassing this value reduce the NADH-dependent fluorescence signal of Frex (Wilkening et al., 2017), in line with the NADH/NAD⁺ dependence of the parental Rex protein reported previously (Gyan et al., 2006; Larsson et al., 2005; McLaughlin et al., 2010). In living *Ralstonia eutropha* cells, Frex shows its full dynamic range (determined for the purified sensor protein upon excitation at 480 nm (Wilkening et al., 2017)) under conditions of sufficient H_2 supply. This indicates that the NAD⁺ concentration is lowered to below 100 μ M by the activity of the SH, since larger NAD⁺ concentrations would limit the dynamic range of the sensor (Wilkening et al., 2017). In our previous work, we estimated that the intracellular NAD⁺ concentration in *R. eutropha* under aerobic conditions corresponds to 1.9 mM (Tejwani et al., 2017). On the one hand, one could infer from this that the maximal Frex fluorescence under aerobic conditions in *R. eutropha* must be affected by the basal NAD⁺ levels and that quantitative NADH determination is not straightforward (Bennett et al., 2009; Tejwani et al., 2017; Zhou et al., 2011). On the other hand, the fact that H_2 treatment (and anoxic conditions) induce(s) the maximum dynamic response of the sensor, at least indicates that the activity of the SH diminishes the free intracellular [NAD⁺] to (less than) 100 μ M with a concomitant increase in free [NADH] into the hundreds of μ M range.

A drawback to the application of Frex in living cells is that only relative changes of its fluorescence amplitudes can be evaluated, while the dynamic range is influenced by several

factors including $[\text{NAD}^+]$. While our data show that Frex can be utilized to measure intracellular $[\text{NADH}]$ changes in bacterial cells grown to the stationary phase, it would be of major interest to monitor the NADH/NAD^+ redox state during the exponential growth phase. However, in a growing culture, the amount of Frex sensor per volume of cell suspension increases in a complicated manner dependent on cell density, and, concomitantly, the fluorescence signal increases in such a way that a correlation with the cellular $[\text{NADH}]$ level is impossible. To resolve this, a signal normalization procedure would be necessary to allow for calibrating to the total amount of sensor protein in the sample, e.g. by utilization of an appropriate fluorescence standard, as in the Peredox sensor (Hung et al., 2011), which carries an additional red-emitting fluorophore for this purpose. In Frex, such an intrinsic normalization procedure would theoretically be possible by utilizing excitation within the other excitation band of the cpYFP chromophore (420 nm). However, the implementation of such a concept entails other experimental difficulties, since the fluorescence signal at 420 nm is much weaker and strongly overlaps with the cellular autofluorescence (Zhao et al., 2011). Thus, to avoid ambiguities due to the variable expression level of the sensor in cells, signal normalization could be facilitated by fusing a red fluorescent protein to Frex. Another point for improvement of the signal intensity would be to exchange the rather poor cpYFP fluorophore by a different one with a higher inherent brightness. Moreover, genetic engineering could be applied to develop sensor variants with further reduced $[\text{NADH}]$ sensitivity, with eliminated NAD^+ sensitivity, or with sensitivity to the $[\text{NADH}]/[\text{NAD}^+]$ ratio.

Even though the Frex sensor has inherited a profound pH-sensitivity from its precursor cpYFP (Day and Davidson, 2009; Schwarzländer et al., 2014; Zhao et al., 2011), it could be ruled out that the sensor's response to H_2 treatment of strain HF798 was significantly influenced by concomitant pH changes in the cytoplasm (Fig. 4). In line with previous observations for mammalian cells (Zhao et al., 2001), this observation indicates that cellular pH regulation in bacteria is under tight control even if large changes in the availability of metabolites are induced.

Despite certain limitations in quantitative studies, the robustness of the fluorescence signal makes the Frex sensor a promising candidate for biotechnological applications to monitor, optimize, or adjust bacterial culture conditions. Preliminary work on this approach is currently in progress and will be the subject of forthcoming studies. Future research might also utilize Frex to monitor SH activity and to correlate this information with insights from established

spectroscopic approaches such as IR or EPR spectroscopy in order to gather comprehensive information in living cells regarding the active states of NAD(H)-coupled hydrogenases, their reaction mechanism, and the parameters controlling their activity.

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Supplementary Information to:

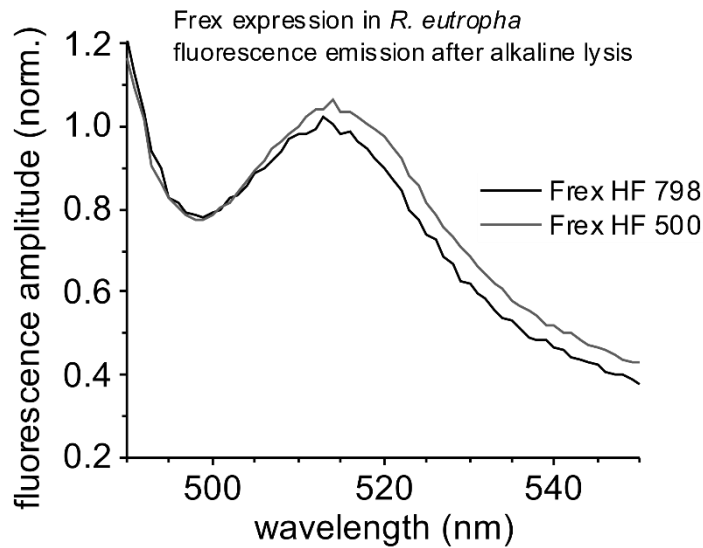
Discriminating changes in intracellular NADH/NAD⁺ levels due to anoxicity and H₂ supply in *R. eutropha* cells using the Frex fluorescence sensor

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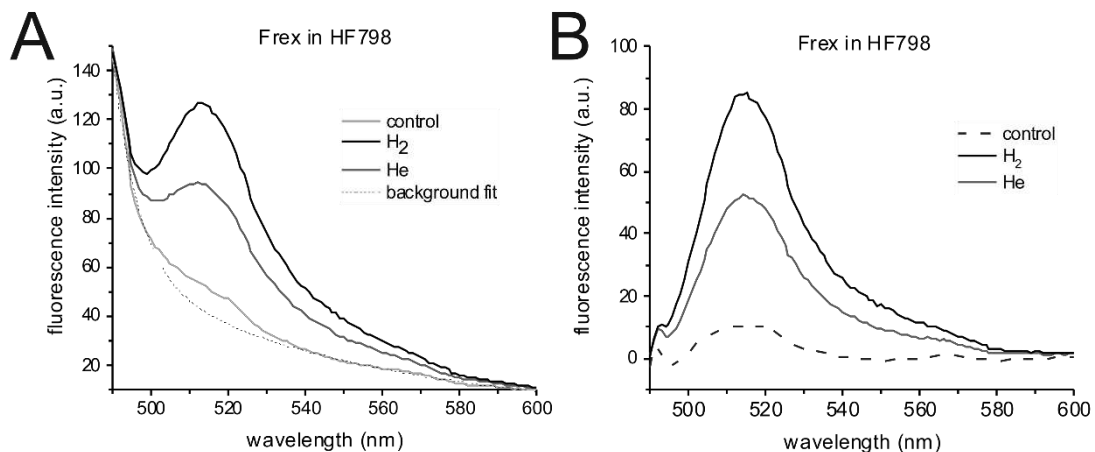
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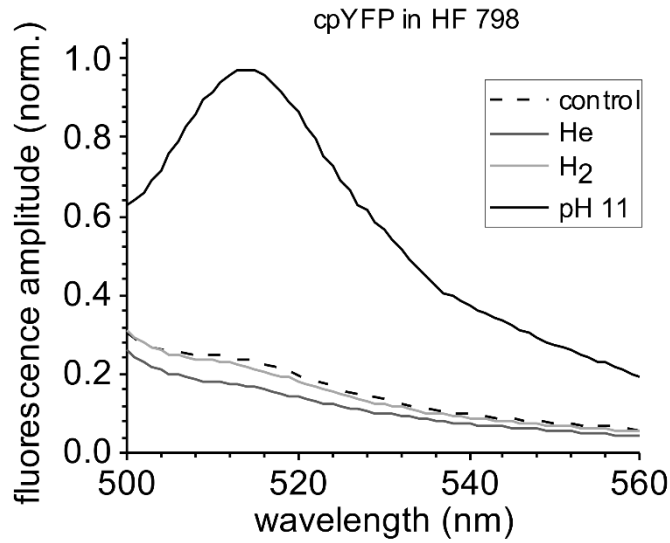
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Supplementary Figure 1: Test for expression of Frex in *R. eutropha* strains upon alkaline cell lysis by treatment with pH 11 buffer. Frex fluorescence was excited at 480 nm, and spectra were normalized to the maximum of the Frex signal from the HF 798 strain at 512 nm for comparison.



Supplementary Figure 2: Procedure to subtract non-specific background from fluorescence signals of Frex-expressing *R. eutropha* cultures. Panel (A) shows raw fluorescence spectra of a cell suspension without and with gas treatment; high background due to light scattering by cells and their components is clearly visible. To compensate for this background, we calculated a biexponential curve (“background fit”, dotted line) by fitting the data of the “control” trace (representing the spectrum of the cell suspension before gas treatment) below 500 nm and above 540 nm with a biexponential function. Fitting by a biexponential curve yielded a better approximation than a typical λ^{-4} scattering function. Subtraction of the “background fit” curve obtained in this way from the raw spectra allows selecting the Frex fluorescence signal around 510 nm from the scattering background. Panel (B) shows the resulting background-subtracted spectra, which correspond to the curves shown in Figure 1A of the main text.



Supplementary Figure 3: Fluorescence spectra from *R. eutropha* cells (strain HF798) expressing cpYFP and lysates derived thereof after excitation at 480 nm. The fluorescence before (dashed line) and after alkaline lysis in buffer with pH 11 (black) as well as after treatment of intact cells with H₂ (light gray) or helium (gray) are shown.

Correlation of elevated Frex fluorescence with hydrogenase activity

To compare the times of elevated (saturated) Frex sensor fluorescence observed in experiments on *R. eutropha* cell suspensions in buffers with different H₂ concentrations (Fig. 2) or with different cell densities (OD₆₀₀ values, Fig. 3) to the activity of the SH, we adapted a hydrogenase activity assay described by B. Friedrich and coworkers [1] for application to permeabilized *R. eutropha* cells. To this end, concentrated *R. eutropha* cell suspensions of a certain OD₄₃₅ were diluted in a H₂-saturated buffer containing 1 mM NAD⁺, which was supplemented with the surfactant cetyltrimethylammonium bromide (CTAB) to permeabilize the cell membranes. The metabolic activity of hydrogenase in the presence of H₂ and NAD⁺ was monitored as the increase of the NADH-specific absorbance at 365 nm based on an extinction coefficient of $\epsilon_{365} = 3400 \text{ M}^{-1} \text{ cm}^{-1}$.

In detail, 5 μL of a concentrated cell suspension with an OD₄₃₅ of 118 were diluted in 2 mL H₂-saturated, CTAB-containing buffer, resulting in an OD₄₃₅ of 0.295. The hydrogenase activity per OD₄₃₅ of the initial cell suspension can be calculated as follows:

$$A_{(\text{OD}_{435})} = \frac{\frac{\Delta\text{OD}_{365}}{\Delta t} \cdot V_t}{\epsilon_{365} \cdot d \cdot \text{OD}_{435}}$$

The increase of NADH extinction in time $\frac{\Delta\text{OD}_{365}}{\Delta t}$ was calculated to 0.016 min^{-1} , the total volume V_t of the cell suspension was 2 mL in a cuvette with a path length (d) of 1 cm, which results in

$$A_{(\text{OD}_{435}=1)} = \frac{0.016 \text{ min}^{-1} \cdot 0.002 \text{ L}}{3400 \text{ L mol}^{-1} \text{ cm}^{-1} \cdot 1 \text{ cm} \cdot 0.295} = 31.9 \text{ nmol min}^{-1}$$

Thus, under the crude assumption of constant enzyme activity, 2 mL of a *R. eutropha* cell suspension (lysate) of OD₄₃₅ = 1 would produce (consume) 31.9 nanomoles of NADH (H₂) per minute. Since in *R. eutropha* cell suspensions of an OD₄₃₅ of 1 corresponds to an OD₆₀₀ of 0.5 (as applied in our experiments with different H₂ partial pressures), and we also used 2 mL samples of cell suspensions, the activity value of $31.9 \text{ nmol min}^{-1}$ can be used to approximate the times needed to consume a certain H₂ concentration¹.

We are aware of the fact that the assumption of a zero-order reaction (constant enzyme turnover) over the whole period of H₂ consumption by hydrogenases is a rough approximation, and that the calculated times for H₂ consumption represent only crude lower limits, since the concentration dependence of enzyme activity implies that the rate of H₂ consumption decreases over time. However, considering the complex reaction balance in the cell lysates used here, a precise model including all (partly unknown and probably counteracting) factors is currently out of reach. Moreover, reactions consuming (producing) NADH in the cell may also exert an influence on the duration of the Frex signal so that the

¹From the values stated here, the hydrogenase activity per cell for a cell suspension of OD₆₀₀ = 0.5 (1 mL of a bacterial suspension with OD₆₀₀ of 1, or 2 mL with OD₆₀₀ of 0.5, correspond to about 10^9 cells) can be calculated as $3.2 \cdot 10^5 \text{ s}^{-1}$, which, together with the assumption of a hydrogenase turnover rate of about 150 s^{-1} [2], would result in an estimate of 2200 hydrogenase molecules per cell, which – in terms of the order of magnitude – matches well with the number of 400 to 2000 [NiFe]-hydrogenase molecules determined by ICP-MS in the similarly sized cyanobacterium *Synechocystis sp.* PCC 6803 [3].

time of elevated Frex fluorescence, during which the NADH concentration is saturating for Frex fluorescence, would represent a lower limit for NADH production by hydrogenases as well. Therefore, the *ad hoc* approach taken here represents a zero-order approximation that needs to be taken with caution.

The H₂ concentration of a saturated aqueous solution at standard ambient temperature and pressure (SATP) can be calculated using the corresponding Henry constant of H₂ gas ($K_H=1282.05 \text{ L}\cdot\text{atm}\cdot\text{mol}^{-1}$), which results in a concentration of about 800 μM H₂ gas at 100 % saturation. With this value and the metabolic activity stated above, one can approximately calculate the time t_{calc} , which a *R. eutropha* cell suspension of a given OD₆₀₀ and volume (here 2 mL) would need to metabolize a given [H₂], as denoted in the following Supplementary Table 1 in comparison to the time of elevated Frex fluorescence (t_{exp}) from Fig. 2B:

Supplementary Table 1:

Volume percent H ₂	n(H ₂) in 2 mL (in nmol)	t_{calc} (in min)	t_{exp} (in min)
10	160	5.0	7.2
20	320	10.0	9.1
30	480	15.0	10.7
40	640	20.1	17.3
50	800	25.1	25.8 [#]

Similarly, the times needed to consume H₂ from a 50 % H₂-saturated buffer by *R. eutropha* suspensions of different OD₆₀₀ (Fig. 3) can be calculated as listed in Supplementary Table 2, as compared to the observed times of elevated Frex fluorescence (t_{exp}):

Supplementary Table 2:

OD ₆₀₀	Activity A (in nmol min ⁻¹)	t_{calc} (in min)	t_{exp} (in min)
1	63.8	12.5	6.9
0.75	47.9	16.7	10.4
0.5	31.9	25.1	20.9 [#]
0.1	6.4	125	23.7

The t_{exp} values marked with “#” in the Supplementary Tables 1 and 2 above were determined under identical conditions. The determined values vary by about 5 min providing an estimate of the experimental error. Considering this magnitude of the experimental error, the calculated reaction times correlate quite well with the theoretical reaction times, indicating that the

elevated Frex fluorescence signal serves as a measure of total hydrogenase activity in living *R. eutropha* cells. A substantial deviation is apparent for the very dilute sample with low cell density (OD₆₀₀ of 0.1) in Supplementary Table 2, in which the calculated reaction time is much larger than the observed reaction time. This deviation might be attributed to substantial effusion of H₂ from the solution (into the essentially H₂-free atmosphere) considering a half-life time for H₂ in aqueous buffer of about 120 min (which would, of course, be modified by the geometry of the container, agitation, temperature etc.). Thus, the experimentally determined reaction times of up to 25 min are not seriously affected by H₂ effusion.

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