The fluorescence-activating and absorption-shifting tag (FAST) enables live-cell fluorescence imaging of *Methanococcus maripaludis*

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Abstract

Live-cell fluorescence imaging in methanogenic archaea has been limited due to the strictly anoxic conditions required for growth and issues with autofluorescence associated with electron carriers in central metabolism. Here, we show that the fluorescence-activating and absorption-shifting tag (FAST) when complexed with the fluorogenic ligand 4-hydroxy-3-methylbenzylidene-rhodanine (HMBR) overcomes these issues and displays robust fluorescence in Methanococcus maripaludis. We also describe a mechanism to visualize cells under anoxic conditions using a fluorescence microscope. Derivatives of FAST were successfully applied for protein abundance analysis, subcellular localization, and determination of protein-protein interactions. FAST fusions to both formate dehydrogenase (Fdh) and F₄₂₀-reducing hydrogenase (Fru) displayed increased fluorescence in cells grown on formate containing medium, consistent with previous studies suggesting increased abundance of these proteins in the absence of H₂. Additionally, FAST fusions to both Fru and the ATPase associated with the archaellum (FlaI) showed membrane localization in single cells observed using anoxic fluorescence microscopy. Finally, a split reporter translationally fused to the alpha and beta subunits of Fdh reconstituted a functionally fluorescent molecule in vivo via bimolecular fluorescence complementation. Together, these observations demonstrate the utility of FAST as a tool for studying members of the methanogenic archaea.

Importance

Methanogenic archaea are important members of anaerobic microbial communities where they catalyze essential reactions in the degradation of organic matter. Developing additional tools for studying the cell biology of these organisms is essential to understanding them at a
mechanistic level. Here, we show that FAST, in combination with the fluorogenic ligand HMBR, can be used to monitor protein dynamics in live cells of *M. maripaludis*. Application of FAST holds promise for future studies focused on the metabolism and physiology of methanogenic archaea.
Introduction

Methanogenic archaea (methanogens) are responsible for producing the majority of methane on Earth and are model organisms for studying cellular processes in the Archaea. Several organisms such as *Methanococcus maripaludis*, *Methanosarcina* spp., and *Methanothermobacter* spp. have been used extensively in genetic or biochemical studies to understand the physiology and metabolism of methanogens (1–3). However, robust tools for direct cell visualization via fluorescence microscopy to allow for protein quantification, analysis of protein-protein interactions, and spatiotemporal characterization of cellular proteins have been lacking. A strict requirement for growth under anoxic conditions, as well as background autofluorescence due to the presence of the oxidized electron carrier coenzyme F$_{420}$ (excitation peak centered at 420 nm, emission peak centered at 480 nm (4)), have prevented the use of most fluorescent protein tags. Green fluorescent protein (GFP) derivatives such as EGFP and mCherry require oxygen for fluorophore maturation and fail to perform under anaerobic growth conditions (5). Alternative oxygen independent probes such as FMN binding fluorescent proteins (e.g. LOV-based fluorescent proteins) typically have weak fluorescence intensity and often exhibit emission around 450 nm, overlapping with excitation/emission spectra of F$_{420}$ (6).

Immunofluorescence strategies using antibody-reporter conjugates typically involve aerobic steps and/or permeabilization of cells using harsh detergents, which preclude live-cell imaging of anaerobes. Another consequence of aerobic preparation of methanogenic organisms is that it causes the rapid oxidation of coenzyme F$_{420}$ which leads to increased autofluorescence.

Recently, an oxygen independent fluorescent protein reporter known as the fluorescence-activating and absorption-shifting tag (FAST) has been developed and adapted in select anaerobic organisms (7–9). FAST is an engineered variant of photoactive yellow protein with a
molecular mass of 14 kDa (7). Like GFP, FAST can be translationally fused to the N- or C-terminus of a gene of interest. By itself, FAST does not exhibit fluorescence; however, upon the addition of a fluorogenic ligand (fluorogen), the two complex together producing a fluorescent product. Multiple fluorogens consisting of 4-hydroxybenzylidene rhodanine (HBR) derivatives exist with each having unique excitation and emission properties upon binding to FAST, and variants of FAST have been developed that specifically bind a subset of fluorogens allowing for two color live-cell imaging (10, 11). HBR derivatives are membrane permeable and bind reversibly and specifically to FAST (7).

There are several reasons FAST is a particularly attractive molecular tool for studying obligate anaerobes such as methanogens. Most importantly, fluorescence is oxygen independent, and membrane permeability of the ligand allows for live-cell imaging. FAST complexes are inherently bright and emit fluorescence across a wide spectrum, avoiding the limitations of reporters such as FMN binding proteins. FAST fluorescence is reversible and quantitative, allowing for direct measurement of relative protein abundance (7, 12). Additionally, splitFAST was recently developed to visualize protein-protein interactions through bimolecular fluorescence complementation (BiFC) (13). Finally, as a small, translationally fused tag, FAST can be used to observe protein localization in live cells.

In this study, we demonstrate successful use of the FAST toolkit in *M. maripaludis*, demonstrating a functional fluorescence-based system for microscopic imaging in a methanogen. We developed a platform for microscopy under anoxic conditions, allowing for visualization of live cells. Combining these advances, we observed robust and quantifiable fluorescence of differentially expressed proteins in cells grown with either H₂ or formate as an electron donor, BiFC using the multisubunit formate dehydrogenase (Fdh), and two different examples of
protein localization. This was accomplished using the original FAST protein, also referred to as FAST1, as well as a highly fluorescent, tandem variant. FAST-based fluorescence microscopy expands existing tools for studying the cell biology of *M. maripaludis* and should be broadly applicable to other methanogens with established protocols for heterologous protein expression.

**Results**

**Expression of FAST1 in *M. maripaludis.***

To test the functionality of FAST, a codon optimized FAST1 gene (14) was expressed in *M. maripaludis* on the replicating vector pLW40 under control of the *Methanococcus voltae* histone promoter (15). Under these conditions, heterologously expressed protein can reach up to 1% of total cellular protein (16). 4-hydroxy-3-methylbenzylidene-rhodanine (HMBR), a FAST fluorogen, was added to stationary phase cultures (OD$_{600}$ = ~0.9) to a final concentration of 10 μM (based on manufacture’s recommendation) before transfer to a 96 well dark plate for quantification on a microplate reader. HMBR fluorescence was measured at 540 nm (excitation with 481 nm). Cultures that were not treated with HMBR exhibited little autofluorescence while cells containing both FAST1 and HMBR exhibited a significant increase in fluorescence (Fig. 1A). Addition of HMBR to wild type cells did not lead to a significant increase in fluorescence compared to cultures expressing FAST1 without HMBR addition.

To further optimize FAST1, we assessed autofluorescence of wild type cells during different stages of growth. We found that cells exhibited the lowest levels of autofluorescence prior to reaching OD$_{600}$ = ~0.40 or ~1.0 in medium supplemented with formate or H$_2$ as the electron donor for growth, respectively (Fig. S1). We hypothesize that increased autofluorescence at higher OD$_{600}$ is due to an accumulation of oxidized F$_{420}$ when electron
donors become growth limiting (17). We also noted that autofluorescence was generally higher in cultures grown on H\textsubscript{2} and lower in cultures grown on formate; therefore, formate grown cells were used in subsequent experiments when possible. Finally, we tested whether altering concentrations of HMBR could further optimize fluorescence over background. In general, increasing concentrations of HMBR led to increased fluorescence (Fig. 1B). For all subsequent experiments, a concentration of 10 µM HMBR was used unless otherwise indicated.

**Anoxic microscopy with a microscope housed inside of an anoxic chamber.**

Due to the oxygen sensitivity of *M. maripaludis*, live-cell imaging requires strict anoxia. To overcome this limitation, we developed a platform to perform fluorescence microscopy using an ECHO Revolve R4 hybrid microscope inside of a Coy anaerobic chamber (Fig. 2A). The microscope utilizes a computer tablet camera in place of an eyepiece, and all manipulations can be performed using a touch screen with a capacitive stylus. Using this system, HMBR addition, culture mounting, and imaging can all be performed without introducing oxygen. The microscope was operated in the upright orientation for all experiments.

Wild type *M. maripaludis* and the strain expressing FAST1 were examined both with and without fluorogen treatment (Fig. 2B). Only FAST1 expressing cells showed noticeable fluorescence gain upon HMBR addition. Because preparing samples in the anoxic chamber involves transferring cells from the high H\textsubscript{2} (or formate) concentrations of a culture tube to the low hydrogen atmosphere of the chamber (3%), cells were imaged immediately after preparation to mitigate the possibility of increasing autofluorescence from F\textsubscript{420} oxidation.

**Evaluation of FruA localization and abundance during growth with H\textsubscript{2} or formate.**
The F₄₂₀-reducing hydrogenase (Fru) catalyzes the reversible reduction of coenzyme F₄₂₀ to F₄₂₀H₂ using H₂ as an electron donor and is the primary source of F₄₂₀H₂ for methanogenesis (18, 19). The large subunit of the hydrogenase, FruA, was selected as a test case for analyzing a FAST1 translational fusion. Fru is abundant in the cell, shows differential expression with increased abundance when formate is supplied as an electron donor for growth, and is thought to associate with the cell membrane (19–21). Using allelic replacement mutagenesis (1), two strains were created with FAST1 translationally fused to either the N terminus or C terminus of FruA. Both strains were analyzed during early exponential growth with either H₂ or formate as the electron donor. In general, cells grown in medium with H₂ exhibited greater autofluorescence when observed under the microscope, and this was true for both N-terminal (FAST1-FruA) and C-terminal (FruA-FAST1) fusions, consistent with the autofluorescence pattern of wild type cells.

The FAST1-FruA construct displayed a pattern of fluorescence consistent with the known membrane localization of Fru (19–21); however, this differed significantly from the pattern observed for the FruA-FAST1 fusion construct (Fig. 3A). Fluorescence in the C-terminal fusion was uniform across the cell while the N-terminal fusion exhibited distinctly higher fluorescence along the outer perimeter of the cell. Distinct foci were regularly observed in the N-terminal fusion strain. The different fluorescence pattern observed between the two FruA constructs is likely due to proteolytic cleavage of the nascent peptide, which is required for maturation of [Ni-Fe] hydrogenases (22). During maturation, Fru is likely proteolytically cleaved at the C terminus, resulting in the loss of the FAST1 tag from FruA-FAST1 fusions and retention of the tag in FAST1-FruA fusions (Fig. 3B). This cleaved FruA-FAST1 expressing strain likely
retains FAST1 in the cytoplasm, leading to uniform cellular fluorescence, while the mature, processed, and nonfluorescent hydrogenase localizes to the membrane.

The FAST1-FruA fusion strain was further analyzed for differences in protein abundance between cells grown in medium containing H₂ or formate. Several transcriptomic and proteomic studies have suggested that Fru is more abundant in cultures grown under conditions where H₂ concentrations limit growth or when formate is provided as the sole electron donor (23–26). The FAST1-FruA fusion strain was examined by anoxic fluorescence microscopy during early exponential growth (OD₆₀₀ = 0.2 – 0.4). As before, cultures were processed in the absence of oxygen, treated with final concentrations of 10 μM fluorogen, and placed on a glass slide for immediate viewing. To control for autofluorescence, light intensity was measured on a per cell basis in the absence of fluorogen and mean intensities of cells after fluorogen addition were normalized to the autofluorescence baseline. Cells grown utilizing formate as the sole electron donor had 1.76-fold higher fluorescence when compared to cells grown with H₂ (Fig. 3C), consistent with increased levels of Fru protein when H₂ concentrations are low.

Quantifying expression of fdhAB with splitFAST and BiFC.

Fdh is a multisubunit protein that is essential for growth on formate (27, 28); therefore, we selected Fdh for further validation of FAST1 in measurements of protein abundance. Additionally, as FAST1 is amenable to analysis using BiFC to study protein-protein interactions (13), we generated FdhAB fusion constructs containing split versions of the FAST1 protein. FAST1 can be expressed as two nonfunctional pieces, NFAST (composed of the N terminal 114 amino acids of FAST1) and CFAST (composed of the subsequent 10 amino acids). With splitFAST, two interacting proteins can be tagged with NFAST and CFAST, and, if they
colocalize, the pieces will reconstitute in the cell to form a fully functioning protein and
fluoresce upon HMBR addition. *M. maripaludis* contains two isoforms of Fdh (29). Fdh1 was
selected for analysis because strains lacking *fdh1* are incapable of growth with formate (27, 28)
and transcriptomic and proteomic studies suggest that, like Fru, Fdh1 is more abundant in
cultures grown under conditions where *H*₂ concentrations limit growth or when formate is
provided as the sole electron donor (23–26).

An *M. maripaludis* strain with translational fusions encoding FdhA-NFAST and FdhB-
CFAST was generated by allelic replacement of the endogenous genes. We additionally
generated control strains containing either FdhA1-NFAST and Mtd-CFAST (Mtd is the F₄₂₀-
dependent methylenetetrahydromethanopterin dehydrogenase) or FdhB1-CFAST and Mtd-
NFAST. Mtd was chosen as a negative control for these studies as Mtd and Fdh are not known to
interact and the genes encoding these two proteins exhibit similar patterns of expression (23, 26). Strains containing FdhA1-NFAST and FdhB1-CFAST, FdhA1-NFAST and Mtd-CFAST, or
Mtd-NFAST and FdhB1-CFAST were analyzed by anoxic fluorescence microscopy. Relative
fluorescence was measured on a per cell basis. Average fluorescence intensity was significantly
higher in the strain containing FdhA1-NFAST and FdhB1-CFAST, and both control strains with
Mtd fusion constructs displayed similar levels of background fluorescence (Fig. 4A and B).

To further validate the use of FAST to measure relative protein abundance across growth
conditions, the FdhA1-NFAST and FdhB1-CFAST containing strain was analyzed for
fluorescence differences between *H*₂ and formate grown cells. In cells grown with formate,
fluorescence intensity was 2.87-fold higher, consistent with increased Fdh abundance when *H*₂
concentrations are low (24) (Fig. 4C).
Use of tandem FAST2 (tdFAST2) to observe cellular localization of FlaI.

In an attempt to further validate FAST for protein localization, we targeted the archaellum (archaeal flagellum) which displays polar localization in intact cells (30). In *M. maripaludis* the major membrane associated components of the archaellum are the anchor (FlaJ) and its associated ATPase (FlaI). Initial attempts to visualize archaella using FAST1 translational fusions were unsuccessful, and we hypothesized that this was due to low fluorescence intensity. To address this, we incorporated a modified version of FAST that has a lower dissociation constant for the fluorogen (FAST2) as a tandem reporter (tdFAST2) (31). This alternative reporter has been shown to achieve higher fluorescence in both bacterial and eukaryotic cells (8, 31).

To test fluorescence, a codon optimized tdFAST2 was transformed into *M. maripaludis* under control of the *M. voltae* histone promoter on the replicating plasmid pLW40neo. FAST1, tdFAST2, and WT were grown to OD<sub>600</sub> of 0.9, and fluorescence was analyzed using a microplate reader and black, flat bottom 96 well plates across several concentrations of HMBR (Fig. 1B). Strains expressing tdFAST2 displayed significant increases in fluorescence over cells expressing FAST1. When normalized to OD<sub>600</sub> and controlling for the inherent fluorescence background, microplate reader assays showed that cells expressing tdFAST2 exhibited a 1.9 - 2.1-fold increase fluorescence over FAST1 upon HMBR addition.

A strain was generated expressing FlaI translationally fused to tdFAST2. These cells were viewed by fluorescence microscopy in an anoxic chamber as previously described except that they were treated with 20 μM HMBR. Robust fluorescence was observed associated with the cell membrane and localized to a single focus, consistent with polar localization of archaella (Fig. 5).
Discussion

Several features of FAST proteins make them ideal for studies in anaerobic organisms. These include the minimal manipulation required to achieve robust fluorescence, a suite of fluorogens with excitation/emission maxima across the visible spectrum (10), and several variant proteins (e.g. FAST1, FAST2, tdFAST, splitFAST, etc…) to facilitate a variety of studies (11, 13, 31). Additionally, we found that FAST can be used to assess differential protein abundance across growth conditions, protein localization, and protein-protein interactions. Used in conjunction with fluorescence microscopy under anoxic conditions, FAST enables tracking of protein dynamics in live cells of *M. maripaludis*.

FAST proteins function with a variety of fluorogenic ligands. For example, binding of the HBR derivative 4-hydroxy-3,5-dimethoxybenzylidene rhodanine (HBR-3,5DOM) shifts the properties of FAST1 such that ex/em wavelengths are 518/600 nm (32). While HBR-3,5DOM resulted in functional fluorescent protein in *M. maripaludis*, overall fluorescence was lower relative to that of HMBR (Fig. S2), and we generally observed higher background fluorescence from the culture medium, so HBR-3,5DOM was not used further. Autofluorescence was also an issue with cultures grown on H$_2$ containing medium, which limited sample collection to exponentially growing cells or cells grown on formate. Autofluorescence was likely due to H$_2$ limitation as H$_2$ diffusion is outpaced by cellular consumption at higher density; under H$_2$ limitation, levels of oxidized, fluorescent F$_{420}$ significantly increase (17). Additionally, removal of cultures from a shaking incubator during sample preparation and transfer to the anoxic chamber can impact H$_2$ diffusion, requiring rapid sample preparation and processing.
Autofluorescence was less of an issue in cultures grown with formate, likely because formate is soluble in aqueous medium so diffusion does not limit growth.

FAST could be further optimized for use in *M. maripaludis* with the use of protein variants. A previous study with FAST2 and tdFAST2 in mammalian cell culture showed that these variants could achieve 1.7-fold and 3.8-fold higher fluorescence, respectively, compared to the original FAST1 (31). We found a more modest increase for tdFAST2 in *M. maripaludis* of ~2-fold. FAST2 has identical quantum yield, a very similar molar absorption value, and a lower dissociation constant than FAST1 (31); therefore, it is likely increased fluorescence with tdFAST2 was a result of the tandem nature of the reporter. The performance of tdFAST2 in *M. maripaludis* was consistent with observations in *Eubacterium limosa* where a 1.5-fold increase in brightness was observed (8).

Expression of FAST proteins in *M. maripaludis* could be accomplished using a high expression vector or under control of native promoters on the genome. Generally, total fluorescence was lower when FAST was expressed from native promoters, and cellular fluorescence varied with the use of different promoters. As a result, expression analysis was carried out on single cells using a microscope. For each fused gene, differences in fluorescence were reflective of observed difference in mRNA and protein abundance in previous transcriptomic and proteomic studies (23–26). For Fru, previous studies found that proteins were ~1.7-fold more abundant when formate was provided as the sole electron donor for growth or when H₂ concentrations were growth limiting. The FAST-based fluorescence pattern observed here is consistent with these data; mean fluorescence intensities for Fru were 1.76-fold higher when formate was provided as an electron donor. For Fdh, previous studies suggested that these proteins were 2- to 3-fold more abundant when cultures were grown on formate (33), consistent
with the 2.87-fold increase in fluorescence for formate grown cells observed here. Together, these data validate the use of FAST protein fusions to measure relative differences in gene expression and protein abundance.

Protein localization applications typically require high fluorescence signal to spatially resolve cellular features. While autofluorescence can complicate these analyses, we successfully visualized the subcellular localization of two proteins that were hypothesized to associate with the cell membrane. Archaella in *M. maripaludis* are associated with the cell pole, and tagging FlA with tdFAST2 resulted in a single discrete focus characteristic of the hypothesized localization pattern. Localization of Fru to the cell membrane was previously inferred from immunogold labeling of this protein in *M. voltae* (20) and from biochemical studies where Fru activity was enriched in membrane fractions (19). Here we provide a third line of evidence for membrane association of Fru. It remains unclear whether membrane association is required for Fru activity *in vivo*.

Protein fluorescence can be used to characterize protein-protein interactions using dual reporter constructs via fluorescence resonance energy transfer or using a single reporter via BiFC. We used splitFAST to tag both subunits of Fdh and observed robust fluorescence above background through BiFC. While modest increases of fluorescence above background were also observed in control strains expressing either NFAST or CFAST fused to Mtd, fluorescence from NFAST and CFAST fused to FdhA and FdhB was significantly higher than either control experiment. High background in control strains was likely due to the fact that Mtd is most abundant when cultures are grown with formate (23, 24), and BiFC can occur through transient interactions between abundant proteins.
We have demonstrated the utility of FAST for protein analysis in *M. maripaludis*.

Combined with anoxic microscopy, FAST allows for live cell imaging, protein localization, determining protein-protein interactions, and expression analysis. These tools should be broadly applicable in other methanogenic archaea with established methods for heterologous protein expression and allelic replacement. Alternative fluorogens and FAST reporter proteins may further expand the utility of FAST in these organisms. The application of anoxic fluorescence reporters presented here expands the already robust toolkit for molecular biology studies in the methanogenic archaea.

### Materials and Methods

#### Strains, medium, and growth conditions.

Strains in this study are listed in Table S1 in the supplemental material. *M. maripaludis* was grown on McCas medium at 37°C with agitation, except that sodium sulfide was replaced by ammonium sulfide for liquid medium (34, 35). For growth in liquid medium, cultures were grown in 5 ml volumes in Balch tubes. When H₂ was supplied as the electron donor for growth, Balch tubes were pressurized to 280 kPa with an 80% H₂, balance CO₂ gas mix. When formate was supplied as the sole electron donor for growth, McCas-formate medium was used at 37°C without agitation (36) and Balch tubes were pressurized to 210 kPa with an 80% N₂, balance CO₂ gas mix. For growth on agar plates, 1.5% noble agar was used and cultures were grown in an anaerobic incubation vessel pressurized to 140 kPa with an 80% H₂, balance CO₂ gas mix as described (34). When necessary, the following antibiotics were added to medium at the noted concentrations: neomycin (1 mg ml⁻¹), puromycin (2.5 μg ml⁻¹), or 6-azauracil (0.25 mg ml⁻¹).
Plasmid construction and transformation of *Escherichia coli*

Plasmids and primers are listed in Table S1 in the supplemental material. To generate FAST constructs, a codon optimized version of the gene was synthesized by Integrated DNA technologies. The codon optimized sequences were determined using the JCat codon optimizer (14) with default parameters with *M. maripaludis* selected as the organism. To generate the tdFAST2 construct, it was necessary to alter the sequence of the gene to prevent loss of the tandem sequence through homologous recombination. To accomplish this, the N-terminal copy of FAST2 was codon optimized in JCat using *M. maripaludis* as the selected organism and the C-terminal copy was codon optimized in JCat using *Methanocaldococcus jannaschii*. The nucleotide sequences of the FAST1 and tdFAST2 genes used in this study can be found in table S2. To further prevent homologous recombination within the tdFAST2 sequence, the 21 base pairs that separate the N-terminal and C-terminal copies were manually edited to reduce similarity to the 33 base pair linker that was consistently used for tagged constructs. In this case, the second most prevalent codon was chosen as determined by the Kazusa database (37).

To generate in-frame FAST constructs, genomic regions flanking either the N terminus or the C terminus of the gene of interest were amplified by PCR using primers suitable for downstream assembly using Gibson assembly (38). Codon optimized FAST1 or tdFAST2 with an additional 33-base pair linker sequence encoding a linker peptide (7) was also amplified by PCR. PCR products were assembled into XbaI/NotI digested pCRuptneo (36). pCRuptneo contains features for propagation in *E. coli* (origin of replication and ampicillin resistance gene) and for selection (neomycin selection) and counterselection (uracil phosphoribosyltransferase) in methanogens. For expression on pLW40 (15), FAST1 or tdFAST2 were PCR amplified and
placed into the vector by Gibson assembly at the NsiI and AscI restriction sites under control of the *M. voltae* histone promoter.

Gibson assembled constructs were transferred to *E. coli* DH5α by electroporation. *E. coli* transformants were selected on lysogeny broth agar medium containing ampicillin (50 μg ml⁻¹).

Plasmids were extracted using the PureLink Quick Plasmid Miniprep kit (Invitrogen) before transfer to *M. maripaludis*. All constructs were sequence verified by Sanger sequencing at the University of Minnesota Genomics Center.

**Transformation of *M. maripaludis***

All strains used in this study were generated in an *M. maripaludis* background lacking the gene for uracil phosphoribosyltransferase (*Δupt* mutant) (34). DNA was introduced into *M. maripaludis* using either a natural transformation method or a polyethylene glycol (PEG) mediated transformation method (1, 39). For splitFAST constructs, compound, sequential transformations were performed using the integrative vector pCRuptneo. NFAST and CFAST sequences were assembled by amplifying a truncated portion of codon optimized FAST1 sequence. Each splitFAST strain was transformed once with either NFAST or CFAST, subjected to selection and counterselection to remove the integrated pCRuptneo vector, then transformed again to incorporate the second tag.

Cultures for natural transformation were grown in McCas medium using a 2% vol/vol inoculum and H₂ as the electron donor. After overnight (~18 h) growth to stationary phase (OD₆₀₀, ~1), cultures were moved into a room temperature Coy anaerobic chamber (3% H₂, 10% CO₂ atmosphere). Plasmid DNA that was preequilibrated for 1 hour in the anaerobic chamber was mixed with 0.5 ml fresh McCas medium and added to the culture using a syringe. The
culture/plasmid mixture was pressurized to 280 kPa with an 80% H₂, balance CO₂ gas mix and incubated at 37°C with agitation for 4 hours. After outgrowth, 0.2 mls of culture material was transferred to medium containing antibiotic (neomycin for pCRuptneo or puromycin for pLW40) to select for transformants. After growth on neomycin, integrative vectors were allowed to resolve by overnight growth without selection. Mutants were isolated by plating onto 6-azauracil containing McCas agar and resulting colonies were screened by PCR.

PEG-mediated transformations followed the protocol of (1, 39) with all steps performed under anoxic conditions. Briefly, cultures were grown to OD₆₀₀ of ~0.7 before washing in transformation buffer (TB: 50 mM Tris, 350 mM sucrose, 380 mM NaCl, 1 mM MgCl₂, pH 7.5). Washed cells were resuspended in 0.375 ml of TB and ~5 µg of plasmid DNA was added before addition of 0.225 ml PEG solution (40% wt/vol PEG 8000 in TB). After a 1 hour incubation, cells were washed twice in McCas medium, pressurized to 280 kPa with an 80% H₂, balance CO₂ gas mix and incubated at 37°C with agitation for at least 4 hours. After outgrowth, cultures were treated the same as for the natural transformation method.

Fluorescence Quantification Using a SpectraMax M2e plate reader

HMBR was purchased from The Twinkle Factory (https://www.the-twinkle-factory.com/) and solubilized in DMSO to a concentration of 2 mM as a stock solution for all experiments. *M. maripaludis* cells were grown in McCas medium with H₂ to stationary phase (OD₆₀₀ = ~0.9) before analysis. *M. maripaludis* liquid culture was directly aliquoted wells of a black, flat bottom 96 well plate (Bioassay systems #P96FL), with 10 µM of HMBR (unless otherwise indicated in the text) to a final volume of 200 µl. Cells were incubated with HMBR for 60 seconds per the manufacturer’s instructions before readings. Fluorescence was measured in a
SpectraMax M2e plate reader (Molecular Devices) and analyzed in SoftMax Pro 7 software. Excitation wavelength was set to 481 nm and emission wavelength was set to 540 nm utilizing auto emission cutoff settings. Each sample was normalized to the baseline reading of the same sample without fluorogen addition to correct for autofluorescence. Samples were further normalized on a per cell basis using OD$_{600}$ of the cells prior to plate preparation.

Samples prepared with HBR-3,5DOM utilized the same methodology as samples treated with HMBR with the addition of a wash step. To reduce the high levels of autofluorescence in the media, cells were pelleted aerobically via centrifugation at 15,500 x g for two minutes and resuspended to their original volume with sterile ddH$_2$O.

**Microscopy**

Imaging was performed in a Coy-type anaerobic chamber with an environment of 3% H$_2$, balance N$_2$. The chamber also contained a 20 cm x 20 cm tray with drierite desiccant to control humidity. All imaging was performed using an ECHO Revolve R4 hybrid microscope operated in the upright orientation with a high resolution condenser (numerical aperture 0.85, working distance 7 mm). Images were taken with the high gain setting on and exposure settings were modified depending on the genetic construct. Cells were viewed using a 40x fluorite objective lens (NA 0.75 and 0.51 mm) or a 100x fluorite oil phase objective lens (NA 1.30 and 0.2 mm). Fluorescence imaging was carried out using standard FITC filter sets.

To minimize autofluorescence, culture tubes were kept in the incubator until they were ready for immediate imaging. Samples were prepared by aliquoting liquid culture into microcentrifuge tubes and subsequently adding HMBR in DMSO to final concentrations as
specified. Aliquots of 5 μl were added onto glass slides and glass coverslips were placed over them. All steps were performed under anoxic conditions.

Quantification of Fluorescence Microscopy Data using ImageJ

For expression analysis, 16 bit .tiff images of both a phase contrast and a fluorescent channel were used to analyze a single field of view utilizing a 40x objective. The FIJI package of ImageJ was used (version 2.1.0/1.53j Java ver. 1.8.0_172). A mask was generated using the phase contrast image by generating an 8-bit image using the threshold tool. The ‘watershed’ and ‘fill holes’ binary features were used as appropriate. The pixels of the resulting image in which cells were located were assigned a value of 1 and pixels elsewhere were assigned a value of 0 through the use of the division function. This image was multiplied with the corresponding fluorescence image to create a composite image with 32-bit float, and the mean fluorescence intensity per cell was determined using the particle analyzer feature. An average of averages was obtained from the compiled list of mean fluorescence intensities. Relative fluorescence units were determined by measuring the gain of fluorescence in cells after HMBR treatment. The average autofluorescent background was determined on a per-sample basis through the methods listed above and was subtracted from the values obtained after the addition of fluorogen. For protein localization analysis, 16-bit .tiff images of both a phase contrast and a fluorescence channel were used to analyze a single field of view utilizing a 100x objective. For figures 3A, 4A, and 5, a median filter was applied for visual clarity.

Statistical Analysis
Data are presented as means ± standard deviation. Statistical analysis was completed using a two-tailed Student t test using Microsoft Excel software. P values for Fdh splitFAST against negative controls were determined using a one-way ANOVA followed up by a post hoc Dunnett’s test for multiple comparison significance in R (version 3.6.2) (40). Significant differences were considered when P values were <0.05.

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**Figure 1.** *M. maripaludis* expressing FAST is fluorescent upon HMBR addition. **A.** Fluorescence intensities of *M. maripaludis* strains cultivated in McCas medium with H₂ as the electron donor for growth. HMBR was added to a final concentration of 10 μM. **B.** Titration of HMBR in cells expressing FAST1 or the tandem variant tdFAST2 grown in McCas medium with H₂. For both panels, relative fluorescence units were determined by normalizing emission readings from a microplate reader against baseline autofluorescence of the sample without fluorogen. Values were also normalized to the OD₆₀₀ of the culture. Data are averages and standard deviations of triplicate measurements.
Figure 2. Anoxic microscopy of *M. maripaludis* expressing FAST1. **A.** The anoxic fluorescence microscope used in all experiments. **B.** Images of wild type *M. maripaludis* and a strain expressing FAST1 from the replicating vector pLW40. Scale bars = 10 μm.
Figure 3. Cellular localization and expression N- and C-terminal translational fusions of FAST1 to FruA. A. Images of N- and C-terminal FAST fusions to FruA. Scalebars = 2 μm. Arrows indicate locations of fluorescent foci associated with the cell membrane. B. Hypothesized maturation of the FruA peptide in the strain expressing fruA-FAST1. Maturation of FruA cleaves FAST off of the mature hydrogenase. C. Fluorescence intensities of FAST1-FruA cells grown in medium with either formate H₂ as the sole electron donor. Values were obtained by averaging fluorescence intensities of single cells from each sample via microscopy. Relative fluorescence was normalized to correct for autofluorescence in absence of fluorogen. Data are averages and standard deviations from five independent cultures.
**Figure 4.** BiFC and fluorescence of Fdh1 with splitFAST. A. Images splitFAST strains of *M. maripaludis*. Yellow arrows indicate the location of a single cell. Scale bars = 5 μm. B. Fluorescence intensities of cells grown expressing various splitFAST constructs. Data were obtained using microscopy and are means and standard deviation of samples collected at least triplicate. C. Fluorescence intensities of *fdhA*-NFAST + *fdhB*-CFAST expressing cells grown in medium either formate or H₂ as the sole electron donor. Data were obtained using microscopy and are means and standard deviation of quadruplicate samples.
Figure 5. Localization of FlaI tagged with tdFAST2. Images are of early exponential phase cells treated with 20 μM HMBR. Scale bars = 5 μm.
References


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