Imaging bacterial protein expression using genetically encoded RNA sensors

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The difficulties in imaging the dynamics of protein expression in live bacterial cells can be overcome by using fluorescent sensors based on Spinach, an RNA that activates the fluorescence of a small-molecule fluorophore. These RNAs selectively bind target proteins and exhibit fluorescence increases that enable protein expression to be imaged in living Escherichia coli. These sensors are key components of a generalizable strategy to image protein expression in a single bacterium in real time.

Protein levels are regulated by complex patterns of synthesis and degradation that may be lost with overexpressed tagged proteins. Imaging of endogenous proteins is an alternative method for tracking proteins that has the potential to provide insights into physiologic expression patterns.

We recently described a strategy to image metabolites using genetically encoded fluorescent sensors composed of RNA1. This method involves fusing metabolite-binding RNA aptamers to Spinach, a 98-nucleotide RNA that switches on the fluorescence of 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), an otherwise nonfluorescent small molecule. The metabolite-binding aptamer is fused via a stem required for Spinach-DFHBI fluorescence. Most aptamers are unstructured before binding their cognate ligand3. Thus, the stem does not form a stable duplex at the imaging temperature. Only after metabolite binding does the aptamer fold, bringing the strands of the stem in proximity, which results in a Spinach structure that can bind DFHBI. The stem sequence that connects the target-binding aptamer to Spinach functions as a ‘transducer’ (Fig. 1a). This transducer module transmits the metabolite-binding event to a fluorescence readout.

Because this method can detect a variety of small molecules in living cells1, we considered whether this approach could be adapted to monitor protein levels in bacteria. To test this idea, we fused a streptavidin-binding aptamer4 to Spinach via different transducer stems, each with a different degree of thermodynamic stability. We tested the ability of each RNA to induce DFHBI fluorescence in a streptavidin-dependent manner (Fig. 1b). Several RNAs functioned as streptavidin sensors as determined by a low fluorescence in the absence of streptavidin and an increase in fluorescence upon streptavidin binding. The optimal sensor contained a transducer module with several base mismatches and exhibited a 10.3-fold fluorescence increase following the addition of 1.7 μM streptavidin (stem 3; Fig. 1b and Supplementary Fig. 1a,b).

We next fused a thrombin-binding aptamer5 to Spinach and generated RNAs with transducer stems of various stabilities as described above. A short transducer module with only two base pairs resulted in optimal protein-induced fluorescence (Supplementary Fig. 2a). The sensor made with this module exhibited a 6.9-fold fluorescence increase following the addition of 1 μM thrombin (Supplementary Fig. 1c,d).

We then generated a sensor for the MS2 phage coat protein (MCP). For these sensors we used the natural RNA-binding element of MCP, the MS2 MCP-binding element (MS2E)6. We fused this stem-loop sequence to Spinach with various transducer stems and generated a sensor that exhibited a 41.7-fold increase in fluorescence upon addition of 4 μM MCP (Supplementary Fig. 1e,f). The optimal transducer module for the MCP sensor was composed of a truncated stem found in the MS2E (Supplementary Fig. 2b). Each of the sensors was activated by only its cognate protein (Fig. 1c–e, Supplementary Table 1). These data also suggest that different transducer domains should be tested to optimize sensor function.

Next, we asked whether these sensors could quantitatively measure protein concentration in vitro. The streptavidin, human thrombin and MCP sensors all gave linear increases in fluorescence with increasing concentrations of each protein (Fig. 1f–h). For example, the thrombin sensor detected human thrombin present in solutions at the nanomolar range (Fig. 1g). Together these data indicate that sensor fluorescence can be used to measure protein concentrations in vitro.

We next used RNA-based sensors to image protein expression in cells. First, we imaged the streptavidin sensor alone or coexpressed with streptavidin in E. coli BL21 cells. We observed a tenfold increase in fluorescence signal when streptavidin was coexpressed (Supplementary Fig. 3).

We then monitored MCP expression following infection with MS2 phages. We first tested whether MCP induced MCP sensor fluorescence in vitro on a timescale relevant for monitoring infection. Mixing purified MCP with MCP sensor led to half the total fluorescence signal in 1 min (Supplementary Fig. 4), a result indicating that the kinetics of fluorescence are rapid.

We monitored fluorescence in individual E. coli cells following treatment with MS2 phages. F pilus–bearing E. coli harboring the MCP sensor under the control of the IPTG (isopropyl β-d-1-thiogalactopyranoside)-inducible T7 promoter were induced...
for 2 h and then treated with phages at a multiplicity of infection (MOI) of 10. After a short lag of 2–4 min, fluorescence increased linearly over time and typically reached a plateau ~25 min after infection (Fig. 2a). These rates correlate with previous bulk measurements of MCP after infection.

We observed a linear correlation between MCP sensor signal and MCP levels measured by western blot, an agreement indicating that the MCP sensor is usable as a direct readout of protein level (Supplementary Fig. 5). We compared the MCP levels to a standard curve of purified MCP (Supplementary Fig. Sa); on the basis of these measurements, ~6 x 10^4 molecules of MCP were synthesized per cell after infection, for a concentration of approximately 60 ± 4 µM. The linearity of the MCP sensor in sensing 0–60 µM of protein in a cell demonstrated that these sensors have the dynamic range necessary to sense most endogenous untagged *E. coli* proteins. MCP sensor concentration was 120 ± 10 µM (Supplementary Fig. 5b), which is twofold higher than the determined maximal MCP concentration. Because the affinity of MCP to the MCP-binding element is approximately 60 ± 4 µM, it is possible to determine the MCP concentration at any moment over time.

Figure 2 | Visualization of MS2 coat protein (MCP) synthesis in individual cells after MS2 phage infection. (a) Kinetics of MCP synthesis after viral infection. *E. coli* expressing the MCP sensor were infected with MS2 phages at a multiplicity of infection (MOI) of 10. MCP synthesis was monitored as an increase in fluorescence signal over time. Images are pseudocolored to show the fold increase in fluorescence at each time point following infection of MS2 phages. Color scale represents 0- to 20-fold changes (black to red) in fluorescence signal. Scale bar, 5 µm. (b) MCP synthesis kinetics in individual cells. *E. coli* were infected at an MOI of 0.1, and fluorescence was monitored over time. One hundred cells were quantified, and an average rate of fluorescence increase was calculated. The average (mean) values ± s.e.m. are shown (gray). On the basis of this average rate, cells were categorized either as fast (red), normal (black) or slow (blue). AU, arbitrary units. (c) Representative examples of fast and slow cells. Fast and slow cells accumulated MCP with a rate greater and less than 2 s.d. from the mean rate, respectively. Time points following application of MS2 phage are indicated. Scale bar, 3 µm.
advantageous in cases in which the target protein function is altered by aptamer binding.

We carried out infections under several conditions to ensure that signal increase was specific to MCP synthesis. The sensor fluorescence was blocked by protein synthesis inhibitors and was not seen when we used control sensors that do not bind MCP (Supplementary Fig. 6). We next tested whether individual cells exhibited variability in MCP synthesis (Fig. 2b,c). Infections were carried out at an MOI of 0.1 to ensure that each cell was infected with at most one MS2 phage (Supplementary Fig. 6). Quantification of the pattern of MCP accumulation in 100 cells showed that 85% of cells exhibited nearly indistinguishable patterns of accumulation of MCP, with near-maximal levels of MCP at 20 min. However, 11% of cells displayed substantially faster accumulation of MCP, whereas 4% exhibited markedly slower rates compared to the average (Supplementary Fig. 7 and Fig. 2c).

To confirm that the observed difference in MCP accumulation kinetics was not due to variability in sensor expression, we compared the expression of a control protein with that of the MCP sensor signal after infection. For these experiments, we expressed blue fluorescent protein (BFP) and the MCP sensor from a single plasmid. In this case, both BFP and the MCP sensor were under the control of separate T7 promoters. After induction, we observed that BFP signal was highly uniform. In contrast, the MCP sensor fluorescence showed more variability than the BFP signal from the same cells (Supplementary Fig. 6b). As an additional control, we coexpressed BFP and Spinach. In this case, BFP and Spinach signals overlapped almost perfectly (Supplementary Fig. 6c), a result indicating that RNA stability does not play a role in the observed differences in signal from the MCP sensor. These results confirmed that cell-to-cell variability exists in MCP synthesis that is independent of the expression level of the MCP sensor.

Unlike the genetically encoded protein-based sensors that are currently available, the sensors described here are composed of RNA and function by allosteric regulation of Spinach fluorescence. These sensors are readily expressed and highly stable in bacteria properties that make them useful for probing bacterial signaling pathways. Because RNA aptamers that selectively bind to numerous types of proteins, including those that do not have RNA-binding domains or any known intrinsic RNA-binding capability, can be readily generated, the strategies described here should enable the design of sensors that detect a wide variety of proteins for diverse applications. Strategies to prevent instability of small RNAs will be needed for application in eukaryotic cells.

Imaging protein expression is important for optimizing production of recombinant proteins. Optimizing protein yield involves screening mutant or genetically modified bacteria for increased protein expression. However, isolating high-producing cells has been a challenge owing to the inability to quantify protein expression in individual cells. Use of the genetically encoded sensors described is a straightforward approach for identifying bacterial cells that exhibit optimized protein expression.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

W.S., R.L.S. and S.R.J. conceived and designed the experiments, W.S. and R.L.S. performed experiments and analyzed data, and W.S., R.L.S. and S.R.J. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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Online Methods
Preparation of RNA sensors and mutants. Secondary structure prediction was performed using Mfold online software. Mutated and truncated RNAs were created by using ssDNA templates (Integrated DNA Technologies) with the desired mutations or truncations and PCR-amplifying these sequences to create dsDNA templates using primers which included a 5′ T7 promoter sequence. PCR products were then purified with PCR purification columns (Qiagen) and used as templates for in vitro T7 transcription reactions (Epicentre) as described previously.

Linear dose-response curve measurements. Dose-response curves for each sensor in response to the target protein were determined by measuring the increase in fluorescence as a function of increasing target concentration in the presence of a fixed concentration of RNA sensor (200-400 nM) and a fixed concentration of fluorophore (10 μM). Fluorescence measurements were performed using a Perkin Elmer LS-55 fluorescence spectrometer using the following instrument parameters: excitation wavelength, 460 nm; emission wavelength, 501 nm; slit widths, 10 nm (Supplementary Note). Curves were determined using a linear regression analysis in GraphPad Prism 5 software.

MCP sensor activation rates. A solution of RNA sensor (10 nM) and DHFBI (10 μM) was incubated with continuous stirring at 25 °C in a buffer containing 40 mM HEPES, pH 7.4, 125 mM KCl and 50 μM MgCl2. The target protein MCP was then rapidly added to the stirring solutions to 78 μg ml−1 (2 μM), and fluorescence emission was recorded over a 15-min period under continuous illumination at 25 °C using the following instrument parameters: excitation wavelength, 460 nm; emission wavelength, 501 nm; increment of data point collection, 5 s; slit widths, 10 nm. The fluorescence increase was then plotted against exposure time and normalized to the maximum intensity.

Cloning sensors for expression in E. coli. The MCP and streptavidin sensors were PCR amplified with primers containing either EagI or SacII restriction sites on the 5′ or 3′ ends of the sensor sequence, respectively. The sensors were then cloned into a plasmid containing a chimera of the human tRNA Lys3 scaffold, which we previously used for Spinach and Spinach-based metabolite sensors and which has previously been shown to stabilize heterologous expression of RNA aptamers in E. coli. The entire tRNA-sensor construct was then PCR amplified with a forward primer containing a BglII site (underlined) and a T7 promoter sequence (bold), 5′-CAG TAG AGA TCT TAA TAC GAC TCA CTA TAG GCT CGA GGC CCG GAT AGC TCA GTC GG-3′, and a reverse primer containing a BlpI site (underlined), 5′-GAT CAG GCT CAG CTG GCG CCC GAA CAG GGAC-3′. This PCR product was then cloned into a pET28c vector directly upstream of the T7 terminator sequence with BglII and BlpI restriction sites.

Live-cell imaging of the streptavidin sensor. BL21 E. coli cells were transformed to harbor either the streptavidin sensor alone or cotransformed to express both pET28c-tRNA-Streptavidin sensor and pET21a-Streptavidin-Alive.

Cells harboring pET28c-tRNA-Streptavidin sensor alone were grown in LB medium supplemented with 50 μg ml−1 kanamycin and 100 μg ml−1 ampicillin, and cotransformed cells were grown in LB medium supplemented with 50 μg ml−1 kanamycin and 100 μg ml−1 ampicillin. Sensor and protein synthesis were induced by addition of IPTG to 1 mM for 2 h. After induction, cells were adhered and imaged as described below.

Live-cell imaging of MS2 coat protein synthesis. DH5α Turbo E. coli cells (New England Biolabs) were used for infection by MS2 phage and imaging because they harbor the F plasmid necessary for MS2 infection. Cells were transformed with 40 ng of plasmid DNA expressing the appropriate RNA aptamer in the context of a tRNA scaffold in pET28c under the control of a T7 promoter as previously described. Additionally, as this strain of DH5α does not already contain a genomically incorporated T7 RNA polymerase, the streptavidin sensor plasmid was cotransformed with a plasmid harboring the T7 polymerase gene under control of a trc promoter. Cells were grown overnight on LB medium supplemented with 100 μg ml−1 ampicillin and 50 μg ml−1 kanamycin to select for those cells that harbored both plasmids. Single colonies were then picked for inoculation in LB supplemented with 100 μg ml−1 carbenicillin and 50 μg ml−1 kanamycin and grown at 37 °C with shaking to OD600 = 0.4, at which point IPTG was added to a final concentration of 1 mM to induce T7 polymerase and MCP aptamer expression. Growth and induction with shaking was continued at 37 °C for 2 h. 100 μl of culture was then removed, spun down to pellet the culture and resuspended in 2 ml of pH 6.0 M9 minimal medium. A 200-μl aliquot of resuspended culture was then plated on PLL-coated 24-well glass-bottom dishes (MatTek) and incubated for 45 min at 37 °C. Adherent cells were washed twice and then incubated with 200 μM DFHBI in pH 6.0 M9 minimal medium for 1 h at 25 °C. Cells were then treated with MS2 phage (ATCC #15597-B1) with an MOI of either 10 or 0.1, as specified. The titer of infection-competent virus was determined by an agar-overlay plaque-formation assay as previously described. Live fluorescence images were taken with a CoolSnap HQ2 CCD camera through a 60× oil objective mounted on a Nikon TE2000 microscope and analyzed with the NIS-Elements software. The filter set used was a sputter-coated filter cube with excitation filter 470/40, dichroic mirror 495 (long pass) and emission filter 525/50 (Chroma Technology). The experiments in which BF and the MCP sensor were coexpressed were carried out as described above; however, BF and the MCP sensor were coexpressed from the pET-Duet vector (Novagen). For experiments in which tetracycline was used to inhibit protein synthesis, cells were grown, induced and adhered to slides as described above. Cells were incubated with 10 μg ml−1 tetracycline for 10 min before treatment with MS2 phage. Tetracycline remained in the medium throughout the duration of the experiment.

Population analysis of MCP synthesis. Population analysis was carried out using NIS-Elements software. Individual cells in a captured field were manually traced as regions of interest (ROIs) and tracked through the different time points of image capture. Fluorescence intensity was then calculated by dividing the total fluorescence by the calculated volume for each ROI, which gives the mean intensity per unit volume. Volume was estimated using the equation $V = \pi r^2(4/3)(r + a)$, where $V$ is volume, $r$ is radius.
and \( a \) is the side length of the \( E. \ coli \). Fold increase in fluorescence over time was calculated as the ratio of mean intensity at a desired time point and at time zero.

The same brightness-over-volume measurement was carried out for BFP and MCP aptamer signal. After this value was calculated, cells were binned according to their percentage of total signal relative to the average signal, which was normalized to 100%.

**Quantitation of MCP and MCP sensor concentrations.** DH5α Turbo \( E. \ coli \) cells were infected with purified MS2 phage at an MOI of 10. At appropriate time points following infection, cells were measured for whole-cell fluorescence using a Tecan SafireII plate reader or were collected and lysed by incubation at 95 °C for 10 min in SDS sample buffer. For fluorescence measurements, background fluorescence for uninfected cells incubated with 200 \( \mu \)M DFHBI was subtracted from all time points. For analysis by western blot, 0.5 OD units of \( E. \ coli \) in sample buffer per time point were subjected to denaturing PAGE. Samples were then transferred to a PVDF membrane by electrophoresis. Western blot was carried out using a rabbit polyclonal antibody against MCP (Millipore no. ABE76) at 1:5,000 dilution. An anti-rabbit horseradish peroxidase–conjugated antibody (GE Healthcare no. NA9340V) was used at a 1:10,000 dilution to detect signal. Bands from western blot analysis were quantified using Image Lab software v.4.0 (Bio-Rad). Concentration of purified protein was determined by BCA analysis (Pierce). The data presented represent mean and s.e.m. from three independent replicates.

The following calculations were used to measure the approximate concentration of MCP molecules synthesized per cell. Based on quantification, a maximum of 1.3 \( \mu \)g MCP was produced per OD\(_{600}\) unit of infected cells. Use of the conversion 1 OD\(_{600}\) = \( 10^9 \) \( E. \ coli \) cells indicated that 1.3 \( \times \) \( 10^{-15} \) g of MCP were produced per cell. The molecular weight of MCP is 12.5 kDa; thus, 6.2 \( \times \) \( 10^4 \) copies of MCP were synthesized per cell after MS2 infection. Using the general conversion of 1,000 copies per cell equals 1 \( \mu \)M, the estimated maximal concentration of MCP produced per \( E. \ coli \) is 60 \( \mu \)M.

The same conversion was applied to determine the total MCP sensor concentration from northern blot analysis: roughly 120 \( \mu \)M of sensor was present under these conditions. Northern blot analysis was carried out as previously described\(^2\) and probed using a 5’ Cy3-labeled probe against the MCP sensor (5’-GGACC CGTCCTTACCATTTTCATTCAG-3’) (IDT DNA). Bands from northern blot analysis were quantified using Image Lab software v.4.0. The data presented represent mean and s.e.m. from three independent replicates.

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