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Notes & Tips

Single cell-level detection and quantitation of leaky protein expression from any strongly regulated bacterial system

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ABSTRACT

Extremely low levels of "leaky" expression of genes in bacterial protein expression systems can severely curtail cell viability when expressed proteins are toxic. A general method for sensitive detection of such expression is lacking. Here, we present a method based on microscopic visualization of a fluorescent "reporter" protein (RFP–HU-A) constructed by fusing red fluorescent protein (RFP) to the N-terminus of a nucleoid-associated, histone-like DNA-binding protein, HU-A. Localization of RFP–HU-A within nucleoids facilitates detection, quantitation, and characterization of leaky expression at the single-cell level.

The bacterium, *Escherichia coli*, is the most widely used of all microbial cell factories available for the expression and purification of proteins [1]; nearly 70% of all publications associated with successful protein crystallization report the use of this organism for protein production [2]. Many factors favor the use of *E. coli* as a cell factory. These include its ease of genetic manipulation, its short generation time, the general ease of protein recovery and scale-up from *E. coli*, and the low costs of growth media and downstream processing [1,3].

A key problem in heterologous protein expression in *E. coli* is that certain proteins are toxic even when levels of expression are very low [3,4]. Thus, many efforts have been directed towards the design of tightly regulated, plasmid-borne, bacterial expression systems in which expression only occurs on "induction" through addition of an inducer molecule supplied to a growing culture of host bacteria, but not "prematurely," i.e., not before addition of the inducer [5,6]. There is little data in the literature on how each such designed system performs, probably because there is no robust general method for evaluating the extent to which "leaky" expression occurs. This is the particular need addressed in this paper.

Our experimental system involves a combination of the pQE-30 vector (Qiagen) and the M15[pREP4] host *E. coli* strain, together constituting a well-known tightly regulated expression system. The system uses two modes of operator binding by repressors; i.e., there are two lac operator sequences located in the neighborhood of a strong T5 promoter, designed to facilitate higher levels of lac repressor binding in the region of the promoter to ensure tighter control on expression prior to induction [http://kirschner.med.harvard.edu/files/protocols/QIAGEN_QIAexpressionist_EN.pdf]. The pQE-30 vector uses the ColE1 origin of replication [7], and it exists within cells in copy numbers estimated to vary between 20–30 [8] and 50–70 copies per cell [9]. To ensure that there are sufficient copies of lac repressor molecules available within M15 cells to fully inhibit transcription from promoter–operator complexes located on pQE-30 plasmid copies, an additional preexisting plasmid, pREP4, is present within the host strain. From plasmid pREP4, lac repressor is constitutively expressed to maintain high levels of the repressor. In the absence of the inducer, isopropyl β-D-1-thiogalactopyranoside (IPTG),[1] no premature protein expression is expected to occur.

To monitor leaky expression, we used fluorescence microscopic imaging of expressed red fluorescence protein (RFP). It is difficult to detect small amounts of freely diffusing fluorescent molecules against the autofluorescence backgrounds of cells [10]; therefore, we decided to perform imaging of localized (rather than diffused) bacterial intracellular fluorescence because highly localized fluorescence signals are known to be more easily detected and quantitated [11]. We genetically fused RFP to the N-terminus of a highly

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[1] *Abbreviations used:* IPTG, isopropyl β-D-1-thiogalactopyranoside; RFP, red fluorescence protein.

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abundant nucleoid-associated, histone-like DNA-binding protein, HU-A, which participates in the compaction of the *E. coli* genome into a structure known as the "nucleoid." The rationale behind the creation of this fusion, RFP–HU-A, was the expectation that it would retain its DNA-binding characteristics and thus localize the fluorescence signal on the nucleoid, enabling visualization and direct comparison of expression in uninduced cells and induced cells.

The fusion gene encoding RFP–HU-A was generated and cloned into pQE-30 as detailed below. The pQE-30 vector and host M15[pREP4] cells were obtained from Qiagen (all other chemicals were procured from Sigma). The *hupA* gene was amplified from *E. coli* genomic DNA by PCR. The *hupA* gene was fused to the 5′ end of the RFP-encoding gene by performing splicing by overlap extension PCR using the following primers: RFP Forward Primer 1, 5′-AGCTACTGGATCCGTGTCTAAGGGCGAAGAG-3′, RFP Reverse Primer 1, 5′-TCCTCCACCTCCGCTTCCTCCCTTGTACAGCT-3′; RFP Forward Primer 2, 5′-CGGAGGTGGAGGAAGCGGAGGCATGAACAAG A-3′, RFP Reverse Primer 2, 5′-GAATACTCCCGGGTTACTTAACTGCG TCTTTCAATG-3′. Amplicons were generated by performing PCR using the following reactions: (a) Forward Primer 1 and Reverse Primer 2, and (b) Forward Primer 2 and Reverse Primer 1. Amplicons resulting from reactions (a) and (b) were then spliced by using strands from the dissociated amplicons themselves as megaprimers, and using Forward Primer 1 and Reverse Primer 1 to amplify the spliced product with terminal sequences encoding the BamHI and HindIII restriction sites. The fusion product was digested by the two restriction enzymes noted above, and cloned into the similarly digested (empty) pQE-30 vector, transformed into M15[pREP4] cells, and selected on kanamycin and ampicillin containing plates. The use of BamHI and HindIII is likely to facilitate the subcloning of this construct into any vector in which leaky expression requires to be checked. The pQE-30 vector allows any protein to be produced with a 6xHis tag at the N-terminus. Thus, the RFP–HU-A fusion protein was produced through IPTG-induced expression of the transformed M15[pREP4] cells in cultures and purification was carried out using standard metal affinity chromatography under nondenaturing conditions. The version of RFP encoded by the fusion was Tag-RFP-T. This version of RFP is encoded by a gene codon-optimized for expression in *E. coli*. Tag-RFP-T is bright and pH stable [12]. The fusion partner, HU-A, is also a highly soluble protein which naturally forms a dimer. We confirmed through gel filtration chromatography that the fusion protein is dimeric, with no detectable aggregation even after 3 months of storage under refrigerated conditions. Notably, HU-A overexpression is known to have no global effects on transcription of other genes [13].

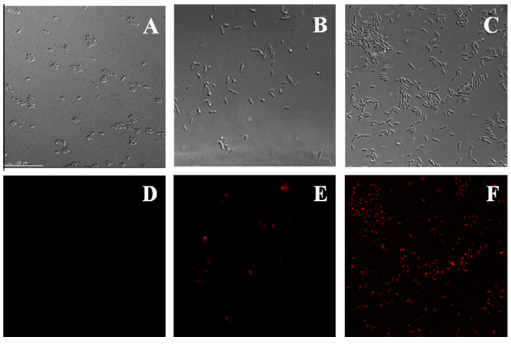

Fig.1. M15[pREP4] cells containing either the empty pQE-30 vector (lacking the insert encoding RFP–HU-A) or the pQE-30 vector bearing the insert encoding RFP–HU-A, imaged using either differential interference contrast (DIC) or fluorescence imaging (shown in red) using the RFP excitation wavelength, for cells that have either been subjected to IPTG induction or deprived of such induction. Panel A shows the DIC image of cells containing empty vector, whereas Panel D shows the same field of cells imaged using RFP excitation. Panel B shows the DIC image of cells containing the vector bearing the insert encoding the RFP–HU-A fusion, without any IPTG induction, whereas Panel E shows the same field of cells imaged using RFP excitation. Panel C shows the DIC image of cells containing the vector bearing the insert encoding the RFP–HU-A fusion, with IPTG induction, whereas Panel F shows the same field of cells imaged using RFP excitation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

RFP–HU-A is fluorescent and binds to DNA. Using the electrophoretic mobility shift assay (EMSA), we confirmed that purified RFP–HU-A protein binds to a standard DNA construct known to bind to HU-A, a four-way junction construct assembled through the association of four separate oligonucleotides (data not shown). Fluorescence emission spectroscopy was also used to establish that the RFP component of the RFP–HU-A fusion retains fluorescence characteristics, by exciting with light of 550 nm and observing the emission spectrum of RFP on a Varian Eclipse spectrofluorimeter (data not shown). The RFP–HU-A fusion thus appeared to be entirely suitable for *in vivo* binding to nucleoid DNA on either leaky or induced expression. Below, we show that RFP–HU-A indeed binds to nucleoids, allowing bacteria expressing it to be imaged using fluorescence microscopy.

M15[pREP4] cells expressing RFP–HU-A from the pQE-30 vector were grown overnight, at 37 °C. Similarly, M15[pREP4] cells containing the empty pQE-30 vector (lacking the insert encoding RFP–HU-A) were also grown. The overnight cultures were then used to inoculate 5 ml of secondary culture, which was grown at 37 °C until the OD at 600 nm reached a value of 0.6. Following this, 1 ml each of uninduced cells was collected and the remaining 4 ml of culture was induced with 1 mM IPTG. Induced cells were allowed to grow for another 4–5 h after which 1 ml was collected. Both uninduced and induced cell samples were centrifugally sedimented into pellets, resuspended in 1 ml of M9 glucose medium, washed, and resuspended in the same medium. About 7 μl of washed and resuspended cells was layered onto agarose pads (made from 1.5% agarose) which were placed on slides and covered with coverslips. Cells were allowed to settle on the agarose pads for 5–10 min prior to imaging.

Cells were imaged using the wide-field, high-resolution, Delta Vision Deconvolution microscope [Model DV Elite, GE Healthcare] equipped with solid state illumination and a 1.4 megapixel monochrome CCD camera [CoolSnap HQ2, Photometrics]. RFP–HU-A control samples lacking pQE-30 plasmid and uninduced and induced samples of M15[pREP4] cells containing pQE-30 were illuminated with 100% transmission of the RFP wavelength output of the microscope's solid state illumination unit (535–561 nm). The imaging was done using a Plan Apochromat 100X/1.4 NA Oil DIC objective (Olympus). The emission filter used to collect images was the TRITC filter (564–611 nm). The exposure time was 0.150 s. The frame size was of 512 × 512, with 2 × 2 binning. False color (red) with intensities proportional to the recorded fluorescence intensities was used to represent RFP–HU-A images. Intensities were quantified using ImageJ software (http://imagej.nih.gov/ij/) by randomly selecting 40 bacteria in each image and measuring individual intensities of fluorescence, subtracting the background fluorescence and averaging to determine the mean fluorescence intensity.

Leaky expression is seen in fluorescence images of uninduced cells. The DNA binding-competent fluorescent protein fusion, RFP–HU-A, binds to DNA and illuminates the whole *E. coli* nucleoid on excitation with light of 535–561 nm. Control panels [showing a representative DIC image (Fig. 1A) and a representative fluorescence image (Fig. 1D)] of cells containing pQE-30 plasmids—but no gene inserts encoding RFP–HU-A—display no fluorescence. Sample panels [showing a representative DIC image (Fig. 1B) and a representative fluorescence image (Fig. 1E)] of cells containing the pQE-30 plasmid, as well as the gene insert encoding RFP–HU-A, but with no induction having been performed through addition of IPTG, however, display some fluorescence signals from some cells, demonstrating that leaky expression occurs to a certain extent in some cells under uninduced (repressed) conditions, with

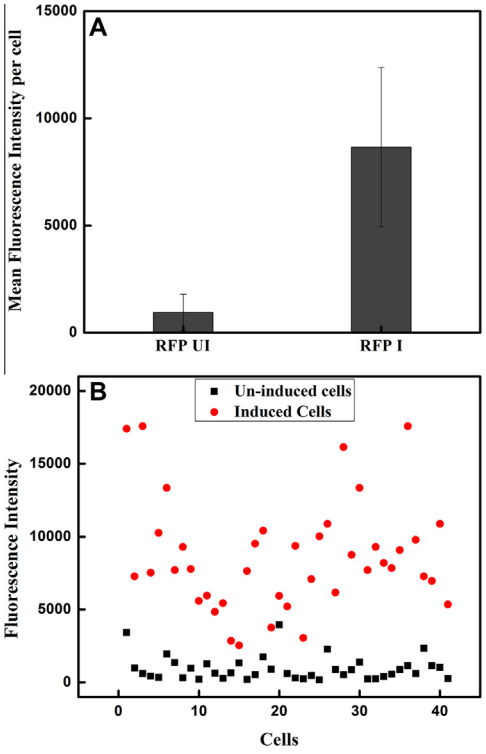

Fig.2. (A) Mean fluorescence intensity per cell seen in the panels in Fig. 1 (Panels E and F). "UI" stands for uninduced cells and "I" stands for induced cells. (B) Scatter plot of intensity of fluorescence from individual cells in populations of uninduced (black squares) and induced (red circles) cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the fluorescence localized around the nucleoid in each cell. Finally, as expected, sample panels [showing a representative DIC image (Fig. 1C) and a representative fluorescence image (Fig. 1F)] of cells containing pQE-30 plasmids as well as the gene insert encoding RFP–HU-A, following induction with IPTG, display evidence of substantial fluorescence from each cell that is seen in the DIC image.

The overall level of leaky expression from pQE-30 in M15[pREP4] is about 6%, with significant cell-to-cell variations. Between the panel showing fluorescence owing to leaky expression without induction for the fusion protein (Fig. 1E), and the panel showing overexpression after induction (Fig. 1F), clear differences can be seen in the intensity of the fluorescence from RFP. In Fig. 2, a quantitation of the imaging data seen in the visual fields in Fig. 1 (obtained using ImageJ software) is presented. Fig. 2A clearly shows (a) that the background-subtracted mean fluorescence intensity seen per uninduced cell is substantially lower than that seen after induction, and (b) also that the fold difference seen between uninduced and induced cells is about 15- or 16-fold for RFP–HU-A. There are noticeable variations in the levels of leaky expression as well as induced expression, from cell to cell and culture to culture. Fig. 2B shows a scatter plot of the intensity of fluorescence seen in each of the 40 cells imaged for the uninduced and induced cultures. The scatter plot establishes three things: (1) the highest intensity seen in any cell in the uninduced population is comparable to the lowest intensity seen in any cell in the induced population; (2) overall, cells in the induced population display much higher intensity than cells in the uninduced population; and (3) in both the uninduced and induced populations, there are large variations in the levels of expression from cell to cell.

The present method allows examination of cell-to-cell variations in leaky and induced expression as well as differences between populations at a very high level of sensitivity; in principle, with the right equipment, the sensitivity could be high enough to allow detection of a single molecule of the fluorescent fusion protein per cell [10], although in the present instance the sensitivity is probably of the level of hundreds of molecules per cell. In contrast, for comparison, a technique like immunoblotting could yield a sensitivity as high as 1–5 pg [14], amounting to about 100 million molecules of a 35 kDa protein like RFP–HU-A. Notably, there appears to be no correlation of leaky expression with either bacterial size or division status. This is to be expected, since even if leaky expression were to result from replication-induced transient derepression, no necessary synchrony would be seen in replication of different repressed gene copies present on different plasmids within the same cell (as replication of plasmids is not synchronous). However, if the experiment had been performed using a single genomic copy of a repressed gene encoding RFP–HU-A, it is conceivable that a correlation could be seen with size or division status.

Many vector–host systems have been developed to address problems arising from protein toxicity. In principle, leaky or low-level expression could occur on account of readthrough transcription from nearby promoters [15] or transient derepression following passage of the replication fork [16,17]. The actual consequences of leaky expression would depend on the required tightness of regulation (which, in turn, could vary from expressed protein to protein, depending on the protein's toxicity).

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