Comparison and Calibration of Different Reporters for Quantitative Analysis of Gene Expression

Hernan G. Garcia

Department of Physics,

California Institute of Technology, Pasadena, CA

Heun Jin Lee

Department of Applied Physics,

California Institute of Technology, Pasadena, CA

James Q. Boedicker

Department of Applied Physics,

California Institute of Technology, Pasadena, CA

Rob Phillips[1]

Department of Applied Physics,

California Institute of Technology, Pasadena, CA

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[1]Corresponding author. Address: Department of Applied Physics, California Institute of Technology, MC 114-96 1200 East California Blvd, Pasadena, CA 91125, U.S.A., Tel.: (626)395-3374, Fax: (626)395-5867

Abstract

Absolute levels of gene expression in bacteria are observed to vary over as much as six orders of magnitude. Thermodynamic models have been proposed as a tool to describe these expression levels as a function of the number and interaction energies of the transcription factors involved in regulating a given transcriptional circuit. In this context, it is essential to understand both the limitations and linear range of the different methods for measuring gene expression and to determine to what extent measurements from different reporters can be directly compared with one aim being the stringent testing of theoretical descriptions of gene expression. In this paper we compare two protein reporters by measuring both the absolute level of expression and fold-change in expression using the fluorescent protein EYFP and the enzymatic reporter β-galactosidase. We determine their dynamic and linear range and show that they are interchangeable over four orders of magnitude. By calibrating these reporters such that they can be interpreted in terms of absolute molecular counts, we establish limits for their applicability: autofluorescence on the lower end of expression for EYFP (at about 10 molecules per cell) and interference with cellular growth on the high end for β-galactosidase (at about 20,000 molecules per cell). These qualities make the reporters complementary and necessary when trying to experimentally verify the predictions from the theoretical models.

Key words: transcriptional regulation; thermodynamic models; beta-galactosidase; fluorescent protein; absolute calibration; fold-change

Introduction

In recent years our understanding of transcriptional regulation has increased dramatically. This is true both in terms of the number of regulatory circuits that have been dissected and of the precision with which they have been characterized (1–7). As illustrated in figure 1, quantitative measurements of gene expression have determined that the mean absolute level of expression of different promoters range over more than six orders of magnitude. The majority of gene products regulated under bacterial and viral promoters are present at levels from 0.1 to 10^5 molecules per cell.

In addition to being concerned with these absolute levels of expression, it is also of interest to know the range over which these promoters can be regulated. As shown in figure 2(C,D) the level of expression of a given promoter can in turn be regulated to vary over several more orders of magnitude. These results make it clear that a quantitative genome-wide characterization of transcriptional regulation requires techniques with a broad dynamic range and for which the experimental uncertainties have been precisely characterized.

Quantitative experiments like those described above are making it possible to directly compare measurements of regulatory response with the predictions of an increasingly sophisticated host of theoretical ideas for describing regulatory circuits (8–17). This poses an experimental challenge: is there a technique or techniques that can reliably span this range of expression?

There are a wide variety of different methods for carrying out measurements of gene expression like those described above (18–21). One classic

scheme for measuring the level of gene expression is based upon the enzyme action of β-galactosidase (LacZ) as a reporter in which a substrate for this enzyme can be detected colorimetrically upon cleavage (18). However, the use of fluorescent reporters is increasingly becoming the method of choice, especially with the construction of a variety of libraries in which nearly each and every gene in a model organism can be read out fluorescently (1, 3, 7). In certain cases, this idea has been pushed all the way to the single-molecule limit where individual molecules in regulatory circuits are detected through their fluorescence (22). mRNA counting both in bulk through quantitative PCR and at the single molecule level is also becoming widespread as a means for quantifying levels of gene expression (7, 23). In the cases where antibodies against the protein of interest are available, Western or immunoblotting can provide a quantitative measure of the protein contents of a cell (24). Finally, another popular enzymatic technique is based on reporting gene expression levels through bioluminescence (19, 25, 26).

The quest to quantitatively dissect regulatory networks of all types (4, 5, 23, 27) raises questions about the relative merits of these different measurement techniques. When trying to compare the significance of these different measurements and to use them as the basis for the development of theoretical models, it is important to have some calibration which reveals their respective dynamic ranges and how they are related. For example, one important question is whether they are linearly related, thus rendering them able to report reliably on the level of expression. Additionally, it is important to determine whether the use of reporters affects cellular processes in any observable way. To that end, in this work, we use systematic ex-

perimentation in the context of a well-characterized regulatory network to compare enzymatic and fluorescent reporters as a measure of level of gene expression. Similarly, recent measurements have begun to systematically explore the relation between the amount of expressed protein and the level of mRNA (7, 23, 28) and the quantification of protein levels through Western or immunoblots (24, 29). Luminescence has an advantage related to its low background levels (30). Being an enzymatic reporter, it requires the addition of a substrate to the medium or the encoding of genes that can produce the substrate within the cell. To our knowledge, even though it has been established that a constant luminescence level per cell can be used to quantify the number of cells in culture with a very high dynamic range (25), only a very limited comparison of luminescence as a reporter for gene expression has been done with respect to another reporter (31). The necessity for providing the cells with a substrate has certainly diminished its use with respect to the widespread fluorescent protein reporters. Additionally, there have been reports of the bioluminescence genes potentially affecting their own expression (32). As such we did not address this technique in this work, though studies similar in spirit to those performed here would be useful.

Our aim was to compare enzymatic and fluorescence reporters for the same promoters in a way that spans the large absolute dynamic range found in bacterial and viral promoters. In analogy to previous work (7, 33) the main strategy consists in engineering a promoter regulated by Lac repressor into *E. coli* which we subsequently induce to a variety of different levels with constructs harboring either a fluorescent or an enzymatic reporter.

The theoretical models mentioned previously predict the fold-change in gene expression, namely, the change in gene expression due to the presence of a transcription factor measured with respect to the level of gene expression in the absence of the same transcription factor as a function of the concentrations and interaction energies of the different molecular players (9, 11, 12). Contrasting such relative predictions with experimental data relies heavily on the linearity of the reporter used. As such, we require not only that reporters span a high dynamic range, but that they also be linear over the fold-change range of the theoretical predictions.

To implement this alternative strategy we constructed a variety of different realizations of the network in which the binding affinities for Lac repressor are varied in a way that leads to different fold-change levels that differ over several orders of magnitude. Using these schemes, we can explore the presumed linearity of response of the enzymatic assays and their fluorescent reporter protein counterparts.

In the following sections we show a comparison and absolute calibration of the two reporters both in terms of their absolute levels and the fold-change in gene expression. We show that they are interchangeable over several orders of magnitude of expression, but each method has a limited dynamic range either due to limitations of the reporter or to how the reporter acts on the cells. We conclude that they are both complementary and necessary if a systematic characterization of the predictions generated by thermodynamic models spanning over multiple orders of magnitude is to be achieved.

Materials and Methods

DNA constructs and strains

The construction of all plasmids and strains if described in detail in the Supporting Materials.

In short, plasmid pZS25O1+11, pZS25O2+11, pZS25O3+11 and pZS25Oid+11 have a *lacUV5* promoter controlling the expression of either a EYFP or LacZ reporter. Care was taken to delete the O2 binding site present in the wild-type *lacZ* coding region (34). These plasmids are shown schematically in figure S7.

A construct bearing the same antibiotic resistance, but no reporter, was created by deleting YFP from one of our previous constructs. This construct serves for determining the cell autofluorescence (for fluorescence measurements) and spontaneous hydrolysis (for enzymatic measurements).

Plasmid pZS3*1-lacI expresses Lac repressor off of a $p_{LtetO-1}$ promoter (35). The ribosomal binding sequence of this plasmid was weakened by mutating it (Alon Zaslaver, personal communication) resulting in pZS3*1BRS1-lacI.

Plasmid pLAU53-NoLacI-TetR-YFP is a derivative from plasmid pLAU53 ((36), kindly provided by Paul Wiggins). It expresses a fusion of TetR to the YFP gene used in this work under the control off the arabinose inducible promoter p_{BAD}.

The *E. coli* strains used in this experiment are shown in table S1. Chromosomal deletions were generated using the protocol developed by Datsenko and Wanner (37).

Chromosomal integrations were performed using recombineering (38). Primers used for these integrations are shown in table S2. The reporter constructs were integrated into the *galK* region (39) of strain HG105 (lacI-) using primers HG6.1 and HG6.3. Strain HG205 (lacI++) was created by integrating pZS3*1RBS1-lacI into the phage-associated protein *ybcN* (40) using primers HG11.1 and HG11.3.

Integrations of the reporters were moved from strain HG105 (lacI-) to strains HG104 (lacI+) and HG205 (lacI++) using P1 transduction (openwetware.org/wiki/Sauer:P1vir_phage_transduction). All integrations and transductions were confirmed by PCR amplification of the replaced chromosomal region and by sequencing.

For YFP measurements of the fold-change in gene expression strains MG1655 and TK140 (4) were used. MG1655 is wild-type *E. coli* encoding the *lac* operon and wild-type levels of Lac repressor. TK140 has a deletion of the *lacI* gene. Unlike strains HG104, HG105 and HG205 these two strains have the *lac* operon, which will result in significant levels of β-galactosidase for strain TK140. As a result, strains MG1655 and TK140 can only be used for fluorescence measurements. Constructs bearing LacZ as a reporter were integrated into strains HG104, HG105 and HG205.

The region of plasmid pLAU53-NoLacI-TetR-YFP covering the p_{BAD} promoter, TetR-YFP and the ampicilin resistance gene was integrated into the *galK* locus of strain H104 using primers HG22.04 and HG22.05 and transduced to strain 563 (kindly provided by Paul Wiggins) to create strain 563::TetR-YFP. This strain has both Tet and Lac repressors binding arrays located at the *lac* operon and near *oriC*, respectively.

All sequences, plasmids and strains are available upon request.

Growth conditions and gene expression measurements

Strains to be assayed for gene expression were grown overnight in 5 ml LB plus 30 μg/ml of kanamycin at 37 °C and 300 RPM shaking. The cells were then diluted 1000 to 4000 fold into 4ml of M9 minimal medium + 0.5% glucose in triplicate culture tubes. Kanamycin was only added at this step for the strains bearing plasmids. The inducer IPTG was also added at this stage if necessary. These cells were grown for 6 to 9 hours until an OD600 of approximately 0.3 was reached after which they were once again diluted 1:10 and grown for 3 more hours to an OD600 of 0.3 for a total of more than 10 cell divisions. At this point, cells were harvested and their level of gene expression measured.

Induction and single cell microscopy was performed on the YFP samples as described in the Supporting Materials. Our protocol for measuring LacZ activity is basically a slightly modified version of the one described in (18, 41). Details are given in the Supporting Materials.

In vivo YFP calibration

Strain 563::TetR-YFP was grown as described in (42), but in the absence of any inducers. In order to reduce the autofluorescence coming from the background buffer/media, we "sandwiched" a small volume of the the cells between two cover glasses corresponding to approximately 25 mm $\times$ 25 mm $\times$ 1 μm. We used low autofluorescence (Corning D263) coverglass and imaged using a 473nm laser in epifluorescence and a EM-CCD Andro iXon camera.

The glass was cleaned using an RCA wash (43). Such a reduction in the background was necessary to get an acceptable signal to noise ratio. The fluorescence of bright spots attributed the EYFP-Tet repressor fusion bound to the DNA was tracked over multiple frames using a customized version of the the Matlab code "PolyParticleTracker" (44). The data was analyzed using custom Matlab code. Representative traces and images are shown in figure 3(A-D). The resulting distribution is shown in figure 3(E).

Results

In the following sections we show a strategy for obtaining an absolute calibration of our two protein reporters. We then compare these reporters side-by-side and determine their ranges of applicability. Finally, we take these experiments one step further by characterizing the fold-change in gene expression measured with both reporters for a simple transcriptional network. This final analysis allows us to determine a range over which thermodynamic models of gene regulation can be tested using this approach.

Absolute calibration of the reporters

Absolute measurements of gene expression are often reported in arbitrary units, especially for fluorescence measurements where the signal depends on the particular details of the microscope used. Such a quantification of fluorescence makes it hard, if not impossible, to compare results between setups and establish unified standards. On the other hand, having a simple way to turn these arbitrary units into an absolute number of molecules

would be helpful both in the context of taking the census of cellular proteins (1, 7, 45) and also in the context of characterizing the limits of each reporter.

In the following sections we obtain an absolute calibration for both the enzymatic and fluorescent reporters characterized throughout this work. In turn, this calibration will allow us to set absolute bounds on the interchangeability of these reporters as well as their effectiveness as reporters of the level of gene expression.

Calibration of the absolute number of EYFP molecules

Several previous experiments have performed absolute calibrations of fluorescence levels by looking at a bulk solution of purified fluorophore in buffer (6, 7, 46, 47) or in cell extract (48). These approaches require a known volume of illumination which can be achieved, for example, by performing either confocal microscopy (46, 47) or two-photon microscopy (6).

These methods should be considered in light of at least two caveats. First, they rely on the extinction coefficient of the fluorophore to determine its concentration. However, the solution will be comprised of active and bleached fluorophores. Therefore the effective extinction coefficient of the solution will be an unknown combination of the extinction coefficients for the active and bleached fluorophores. Second, they are performed outside the cell. Even in the case of cell extract the local environment the protein sees might be different than that of the unperturbed cellular interior.

Counting fluorescent proteins inside the cell is, however, not straightforward. Because of the fast diffusion time of free fluorescent proteins in the cytoplasm (49) the signal of individual fluorescent molecules gets blurred

over the cell on the time scale of tens of milliseconds. As a result the fluorescence per unit area of a single fluorophore in the cytoplasm becomes comparable to the cell autofluorescence and, hence, not detectable under common continuous illumination conditions. A way to circumvent this is by immobilizing the fluorophore. For example, membrane proteins fused to fluorescent reporters present a much slower diffusion on the membrane than that of proteins in the cytoplasm. Single fluorophores can be then imaged in this way (7, 50, 51).

Our main approach for calibrating the fluorescence of a single EYFP molecule consists in immobilizing EYFP molecules *in vivo* by fusing them to a transcription factor which is in turn strongly bound to the genomic DNA of *E. coli*. Though this method has the advantage of being *in vivo*, one caveat is that in this case we are not imaging free cytoplasmic EYFP like in the gene expression measurements in this work. The fact that EYFP is fused to another protein that is in turn bound to the DNA could result in a difference of fluorescence.

Puncta of EYFP fused to Tet repressor could be observed inside the cells despite the poor signal-to-noise ratio of about 1.5. In some cases these puncta could be observed to disappear in a single step as shown in the trace in figure 3(A,B). We associate this with the photobleaching step of a single EYFP molecule. More often though the puncta would correspond to multiple EYFP molecules. These fluorescence traces manifest multiple discrete levels as shown in figure 3(C,D). By integrating the fluorescence of the steps over a small area we can obtain the distributions of steps shown in figure 3(E). Please, refer to the Supporting Materials for a detailed discussion

of the data analysis process.

We compared the fluorescence per EYFP molecule to the total fluorescence coming from a particular strain, HG105::galK<>25O2+11-YFP, under the same conditions. This strain expresses cytoplasmic EYFP. As a result we estimate the number of EYFP molecules in this strain to be $2,600 \pm 600$. The reasoning behind choosing a strain where we directly measure the number of EYFP molecules is that all further gene expression measurements with EYFP as a reporter will be measured with respect to this "reference" strain. In this way we can easily estimate the number of EYFP molecules in any other strain we measure. Finally, we also quantified the fluorescence of single purified EYFP molecules and obtained a consistent result within 15 %. Please, refer to the Supporting Materials for details of this single molecule fluorescence quantification.

As a sanity check on these results, we estimate the expected number of EYFP molecules. The average number of EYFP molecules in steady state can be approximated by

$$\langle \text{EYFP} \rangle = \frac{\alpha \times b}{\beta}, \tag{1}$$

where α is the mRNA production rate, b is the number of proteins made per mRNA molecule, and β is the protein decay rate (52). Because of the long lifetime of EYFP, the "decay rate" is actually nothing more than the cell doubling time since each cell division effectively halves the number of proteins. For the experiments considered here, we have a cell division time of around 1 hour. The number of proteins per mRNA has been measured for the *lac* operon to be about 20 protein molecules per mRNA molecule

(53). However, the *lac* operon has a different ribosomal binding site than the constructs used in this paper. Recently, models to predict the strength of a ribosomal binding site have been developed (54). Such models predict that our ribosomal binding sequence should be about 5.4 times stronger than that of the wild type *lac* operon. Therefore, we take $b = 20$ proteins/mRNA $\times$ $5.4 = 108$ proteins/mRNA. This number is within the range of the various protein/mRNA measurements performed by (7). Finally, the transcription rate for the fully induced *lac* operon has been reported to be between 1 min^{-1} and 0.06 min^{-1} (55, 56). However, the lacUV5 promoter is about 30% weaker than the wild type *lac* promoter (57) resulting in a range of $\alpha = 0.04 - 0.7$ min^{-1}. When combining the decay rate β, the translation rate b and the transcription rate α we obtain an expected number of EYFP molecules of $370 - 6500$ per cell, a range comparable to our measurement.

Calibration of the absolute number of LacZ molecules

A simplified version of the reaction describing the breakdown of ONPG into ONP by β-galactosidase is given by

$$\text{ONPG} + \text{LacZ} \xrightarrow{k} \text{ONP} + \text{LacZ}. \tag{2}$$

From this reaction scheme we can derive the rate equation for the production of the yellow compound ONP which is given in turn by

$$\frac{d[\text{ONP}]}{dt} = k[\text{ONPG}][\text{LacZ}] \tag{3}$$

and a rate equation for the decay in ONPG concentration due to its hydrolysis

$$\frac{d[\text{ONPG}]}{dt} = -k[\text{ONPG}][\text{LacZ}]. \tag{4}$$

We wish to obtain the concentration of β-galactosidase, [LacZ], in our reaction in order to calculate the number of LacZ molecules per cell in the culture that was used to perform it.

If we assume that we have an excess concentration of ONPG and that the time of the reaction is short compared to $1/(k[\text{LacZ}])$ we can neglect its depletion during the reaction. As a result we take [ONPG] as a constant in equation 3. The reaction described by equation 3 is the one we perform in the β-galactosidase assay to measure the amount of LacZ molecules per cell in Miller Units (MU). In this assay we monitor the production of ONP over time given by the increase in absorbance at 420 nm of the solution. The standard definition of the Miller Units (18) is

$$\text{MU} = 1000\frac{\text{OD}_{420} - 1.75 \times \text{OD}_{550}}{t \times v \times \text{OD}_{600}}, \tag{5}$$

where v is the volume of cells used in ml at a cell density given by OD_{600} and t is the reaction time in minutes. These Miller Units were defined such that the fully induced wild-type *lac* operon has an activity of 1000 MU and such that its non-induced level would yield 1 MU approximately. We seek to relate these arbitrarily-defined Miller Units defined in equation 5 to equation 3 in order to obtain an actual number of LacZ molecules inside the cell.

First, the term $\text{OD}_{420} - 1.75 \times \text{OD}_{550}$ in equation 5 is a measure of the amount of ONP, the product of the breakdown of ONPG by β-galactosidase,

in the reaction corrected for the cell debris (see Materials and Methods). We relate the absolute concentration of ONP in the reaction to the absorption reading through this term such that $\gamma[\mathrm{ONP}] = \mathrm{OD}_{420} - 1.75 \times \mathrm{OD}_{550}$. From an experimental point of view, the key assumption is that of a linear increase in the amount of ONP over time. Given that at the moment the experiment starts, there is as yet no ONP, we can obtain $d[\mathrm{ONP}]/dt$ simply by taking the accumulated ONP at time t and dividing by this elapsed time, that is,

$$\frac{d[\mathrm{ONP}]}{dt} \approx \frac{[\mathrm{ONP}]}{t}. \tag{6}$$

We also invoke a relation between the OD_{600} reading and the density of cells such that $\mathrm{OD}_{600} \times v \times \delta = N_{cells}$, where N_{cells} is the number of cells. Finally, we wish to obtain the number of LacZ tetramers present in the reaction N_{LacZ} from this previous equation. This can be done by rewriting the concentration as $[\mathrm{LacZ}] = N_{LacZ}/V$, where V is now the reaction volume of the standard Miller LacZ assay. If we insert this in our definition of MU we get

$$\mathrm{MU} = 1000\,\gamma\,k[\mathrm{ONPG}]\delta\frac{1}{V}\frac{N_{LacZ}}{N_{cells}}. \tag{7}$$

We determined δ for our strains to be $(8.9 \pm 0.8) \times 10^8$ /ml. The relation between ONP absorption at 420 nm and concentration is $0.0045/\mu\mathrm{M}_{\mathrm{ONP}}$ approximately (18, 58). The volume of the reaction in the standard Miller assay is $V = 1.2$ ml. However, before the ONP reading the sample gets diluted to around 1.7 ml by the addition of Na_2CO_3. Therefore we define γ=$0.0045/\mu\mathrm{M}_{\mathrm{ONP}} \times$ (1.7 ml/1.4 ml). Finally, we need to obtain the turnover rate of LacZ given by k. Wallenfels and Weil (59) report a turnover

rate of $138 \times 10^6 \frac{\mathrm{M_{ONP}}}{\mathrm{min} \times \mathrm{M_{LacZ}} \times \mathrm{M_{ONPG}}}$, where we are referring to LacZ tetramers. Similar values have been reported by other authors (56, 60). Since the initial concentration of ONPG in the reaction is 1.86 mM we get

$$\mathrm{MU} \times \mathrm{ml} \times \mathrm{min} \times 0.5 \approx \frac{N_Z}{N_{cells}}. \tag{8}$$

Although this is only a rough estimate because of a lack of error bars associated with the reported values for the specific activity of LacZ, this gives us a direct connection between Miller Units and number of LacZ molecules per cell.

This LacZ calibration that we have just calculated is consistent with previous experimental results on the *lac* operon. For example, the expression level of the repressed operon is about 0.6 MU (4). Our calibration suggests that this corresponds to 0.3 LacZ tetramers/cell. Using single molecule techniques, the average number of LacZ tetramers under repressed conditions was estimated to be 1.2 tetramers/cell (53). The internal consistency of these different estimates is encouraging.

Limits of LacZ and YFP as absolute reporters of gene expression

Recall that our aim was to compare enzymatic and fluorescence reporters for the same promoters in a way that spans the large dynamic range found in natural bacterial and viral promoters.

As we have already noted, the level of expression in such bacterial promoters span over six orders of magnitude as shown in figure 1 and table S3.

Our interest was to design an experiment that would permit us to capture a similar dynamical range in a way that would result in a systematic comparison between the enzymatic and fluorescent reporters. To that end, we use an approach based on induction. We use an inducible *lacUV5* promoter with a single binding site for Lac repressor (Oid) located directly downstream from its transcriptional start (see figure S7). Two versions of this construct regulating the expression of either the *lacZ* or EYFP genes were created. These constructs were either located on the bacterial chromosome or a low copy plasmid in strains that bear wild-type levels of Lac repressor (lacI+), high levels of Lac repressor (lacI++) or no Lac repressor (lacI-). By growing the different combinations of resulting strains at different concentrations of the inducer IPTG we were able to compare the total EYFP fluorescence and LacZ enzymatic activity per cell. These induction curves are shown in figure S8.

In figure 4 we present the corresponding expression levels measured using the two reporters over four orders of magnitude. For comparison, these results are juxtaposed with the literature expression levels of some naturally occurring promoters such as those presented in figure 1 and table S3. The blue line corresponds to a fit to a linear model showing that the data is consistent with a linear relation between the two reporters. This observation is consistent with recently published results (7). The slope or conversion factor is $(9.6 \pm 0.7) \times 10^{-5}$ arbitrary fluorescence units/Miller unit (MU). Even if we fit the relation between the two reporters with a more general functional form such as a power law, we find a linear dependence as shown in figure S10.

Although β-galactosidase activity is measured in absolute units, the fluorescence intensity depends strongly on details of the experimental apparatus used for the measurement such as the illumination intensity and transmission of the optical elements. The calibrations mentioned above that convert YFP arbitrary fluorescent units and LacZ Miller Units into a number of molecules allows for our expression levels to be converted into an approximate absolute number of molecules of each reporter as shown by the labeling on the alternative axes in figure 4. We estimate the EYFP-LacZ relation to be around roughly 0.1 EYFP molecules/LacZ monomer. This value seems to be at odds with the fact that they are being expressed from the same promoter. If the transcription rate is the same because of this then that leaves some difference at the translation initiation and translation levels. However, we lack sufficient information to estimate those differences. Alternatively, an underestimation of the number of EYFP molecules inside the cells could be due to issues related to the fluorescence of the molecule itself such as quenching and misfolding (61).

Fluorescence measurements are fundamentally limited for low levels of gene expression. When the fluorescence signal becomes comparable to the autofluorescence level (below 10 molecules/cell) the determination of the level of gene expression has a high associated error. In contrast to free cytoplasmic fluorescent proteins, this limitation is less stringent in the case of fluorescent proteins that are immobilized on the cell membrane (50, 61) or DNA by a fusion (this work and (62)). The high error in the determination of low expression levels is reflected in the fluorescence distributions shown in figure 5. For the lowest expression levels, the dominant error comes

from variations in the autofluorescence. For example, we observe a slight systematic bias towards overestimating the level of autofluorescence. Given the size of the autofluorescence variation, we do not regard the mean value of fluorescence as statistically significant. This limitation is indicated as a grey shaded area in figure 4. To give a sense of the scale, the expression level of the repressed wild-type *lac* promoter could not be measured with fluorescence unless a more sophisticated technique to visualize single fluorescent proteins is invoked (50). By way of contrast, no significant analogous background was observed in any of our LacZ measurements, showing that this method is more reliable for quantifying very low levels of gene expression. In fact, linearity of the LacZ activity has been reported down to 0.03 MU (4).

When performing a measurement of gene expression using reporters it is important to demonstrate that the presence of the reporter itself is not affecting the state of the cell. We choose the growth rate as an indicator of the cellular state. For all the expression levels shown in figure 4 the doubling rate is approximately one hour regardless of the reporter. However, strain lacI- bearing a plasmid with LacZ as a reporter showed a longer doubling time of (74 ± 1) minutes, which was not the case for the corresponding EYFP strain. These growth rates are shown in table 1 and the corresponding growth curves are shown in figure S11. We confirm previous observations that expression levels above 20,000 LacZ tetramers/cell start affecting the cell significantly (63). Unlike the low end of expression, where EYFP was limited by the autofluorescence, we find that for the high end of expression LacZ becomes limiting not because of signal issues, but because the cell is affected by the fact that high levels of LacZ are being expressed.

Interestingly, even some of the stronger promoters such as rrnB and the T7 A1 promoter have levels below this threshold.

Though our measurements primarily focused on the use of microscopy to quantify EYFP fluorescence it is by no means the only option. An alternative is, for example, to use a plate reader. Though this method does not provide single cell information, it is able to produce data in much higher throughput than microscopy. On the other hand, plate readers will be more limited in terms of the minimum level of fluorescence they can quantify reliably. We perform a comparison between fluorescence measurements on the same strains using microscopy and a plate reader in the Supporting Materials leading to figure S6. We reach the conclusion that they are completely interchangeable, but that the lower limit of detection is now on the order of 50 molecules/cell, roughly 5 times more than with microscopy.

Limits of LacZ and YFP as reporters of the fold-change in gene expression

The fold-change in gene expression due to regulation by a transcription factor is defined as the level of gene expression in the presence of that molecule divided by the level of gene expression in its absence. In particular, it is the key magnitude predicted by thermodynamic models of transcriptional regulation (11, 12). These models can predict fold-changes in gene expression that span over multiple orders of magnitude for both repression (fold-change< 1) and activation (fold-change> 1).

In order to test these models it is then necessary to be able to decide which reporter will be the best to assay a particular type of regulatory

architecture. For example, in the previous section we found that we can reliably measure EYFP fluorescence down to 10 molecules/cell. If we are dealing with a promoter with a basal expression level of approximately $3,000$ YFP molecules/cell like the *lacUV5* promoter integrated on the chromosome used in this work this means that the lowest fold-change we can measure with YFP is $10/3000 \approx 10^{-3}$. On the other hand, the maximum LacZ activity attainable before cell growth starts being compromised is around 20,000 LacZ tetramers/cell. This means that we can only *increase* the number of LacZ tetramers beyond the basal level up to this level before the cell senses the presence of these molecules as measured by its growth rate. Since the basal level of our promoter corresponds to $4,000$ LacZ tetramers/cell this translates into a maximum measurable fold-change of $20000/4000 \approx 10^{1}$ using LacZ as a reporter.

In order to test part of this assertion about the maximum fold-change in repression we performed fold-change measurements on constructs bearing the operators O1, O2 or Oid and the reporters LacZ or EYFP. Figure 6 shows the fold-change measured using EYFP as a function of the fold-change measured using LacZ for the different single binding site constructs (O1, O2 and Oid) in two different Lac repressor backgrounds: lacI+ and lacI++. We see that the fold-change levels measured with both reporters are in good correspondence. As expected, when the fold-change in gene expression reaches 10^{-3} the EYFP readings start becoming too noisy to determine the fold-change in gene expression reliably, setting a limit on the range of fold-change that can be measured using EYFP as a reporter.

Discussion

In this work we explored the feasibility of testing theoretical models of gene regulation using two reporters of protein expression: EYFP and LacZ. The calibration between EYFP and LacZ levels shown in this work is an important methodological prerequisite for testing quantitative models of gene expression. One important outcome is that it makes it possible to compare previously available data, generally taken using LacZ as a reporter, with single cell expression data obtained using EYFP over most of the range of expression of bacterial promoters.

Fluorescent molecules have generally been the method of choice recently because they allow for live imaging of single cells. Our work establishes a clear absolute boundary for these measurements: the autofluorescence level. The intuitive expectation that autofluorescence will contaminate fluorescent gene expression measurements is converted into a concrete and precise numerical criterion. We expect this absolute boundary to be dependent on the particular fluorescent protein used as they can vary widely in their spectral properties and as the autofluorescence is also measurably different at different wavelengths (64). Interestingly, the enzymatic activity of LacZ shows no such limitation. However, for high levels of expression the presence of LacZ affects cell growth in a detectable way before any similar effect from EYFP can be detected. The experimental capacity to use both methods and to switch between one reporter and the other presented in this work makes it possible to obtain the best of both worlds: very low expression levels can be measured accurately in bulk using LacZ in absolute units whereas

slightly higher levels of expression can be measured at the single cell level using fluorescence. Because of fundamental limitations associated with each reporter we conclude that both techniques need to be used together if the full range of absolute gene expression is to be measured. The outcome of this work has direct consequences on the fold-change in gene expression detectable with each reporter and, in turn, on the range of predictions that these measurements can be contrasted against.

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Tables

Strain	**Location**	**Reporter**	IPTG (μM)	Doubling time (min)
lacI++	Chromosome	No reporter	0	59 ± 1
lacI++	Plasmid	No reporter	0	57 ± 1
lacI-	Plasmid	EYFP	0	59 ± 2
lacI+	Plasmid	LacZ	1000	62 ± 1
lacI+	Plasmid	LacZ	0	59 ± 1
lacI-	Plasmid	LacZ	0	74 ± 1

Table 1: Effect of expression level on growth rate. Cells expressing a high level of EYFP (lacI-/Plasmid) have effectively the same doubling time as a strain without any reporter (lacI++/Chromosome/No reporter and lacI++/Plasmid/No reporter). However, the same is not true for high LacZ levels (lacI-/Plasmid), where the level of expression affects the doubling time in a measurable way.

Figure Legends

Figure 1.

Gene expression levels in *E. coli*. The estimated absolute expression level of several bacterial promoters and a strong viral promoter obtained from the literature are shown in red (see Supporting Materials and table S3 for the corresponding references and assumptions made in order to determine the level of expression). For comparison the results from two recent cell censuses of *E. coli* are also shown as histograms of the number of proteins (7, 65). Note that the range of expression spans over about six orders of magnitude for a given set of measurements illustrating the wide dynamical range associated with bacterial promoters. The discrepancy between the two cell census of *E. coli* are further explored in figure S9.

Figure 2.

Fold-change of different regulatory motifs. The states and weights from the thermodynamic models are shown for the case of (A) simple repression by Lac repressor and (B) simple activation by CRP. The corresponding fold-change in gene expression as a function of transcription factor concentration predicted by the model is shown in (C) for repression and (D) for activation. The fold-change values span over several orders of magnitude. Refer to (12) for a derivation of the respective formulas and their parameters which are characteristic of bacterial promoters. The data for Lac repressor in (A) has been taken from (66).

Figure 3.

Absolute *in vivo* fluorescence calibration. Representative fluorescence snapshots (A) and their corresponding fluorescence traces (B) for a single bleaching event of the EYFP-Tet repressor fusion bound to the genomic DNA. (C,D) Snapshots and fluorescent traces for multiple bleaching events of the EYFP-Tet repressor fusion. The red lines correspond to a least squares fit to a single or multiple step function. (E) Distribution of fluorescence of bleaching steps for the *in vivo* sample.

Figure 4.

Relation between the mean cell fluorescence and β-galactosidase activity. The fluorescence per cell is plotted against the $\beta-$galactosidase activity. Each point corresponds to the same construct bearing either EYFP or *lacZ* as a reporter in the same strain background and at the same concentration of IPTG. The blue line is a linear fit fixing the intercept to zero with a slope of $(9.6 \pm 0.7) \times 10^{-5}$ fluorescence units/MU or an estimated 0.1 YFP molecules/LacZ monomer. The grey shaded area represents the range of YFP where the fluorescence signal is comparable to the cell autofluorescence (see discussion in the main text and figure 5). The red shaded area corresponds to the range where our assay can detect LacZ expression affecting cell growth (refer to the main text and to table 1). The expression values of several natural promoters, some of which are also shown in figure 1, are plotted on the blue line.

Figure 5.

Reproducibility of low fluorescence levels. Histograms of the mean fluorescence per area in single cells corresponding to a highly repressed samples and two repeats of the same non-fluorescent control are shown. The variation observed in these samples is comparable to the separation between non-fluorescent and low fluorescent distributions resulting in a considerable error in the estimation of the fluorescence of the sample.

Figure 6.

Fold-change in gene expression measured by LacZ and EYFP. The fold-change of a construct bearing a single Lac repressor binding site (Oid, O1 and O2) in the lacI+ and lacI++ backgrounds is compared when *lacZ* and EYFP are used as a reporter. The line has a slope of one. The point in the plot displaying the lowest fold-change corresponds to fluorescence levels that are near the detection limit. This results in the very large error bar shown.

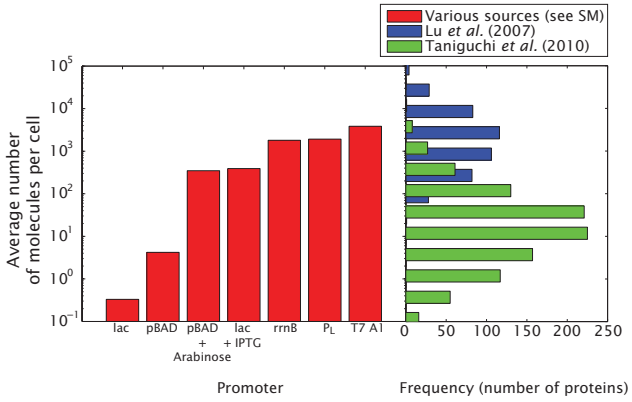
Various sources (see SM)
Lu et al. (2007)
Taniguchi et al. (2010)
Average number of molecules per cell
10^5
10^4
10^3
10^2
10^1
10^0
10^{-1}
lac
pBAD
pBAD + Arabinose
lac + IPTG
rrnB
P_L
T7 A1
0
50
100
150
200
250
Promoter
Frequency (number of proteins)

Figure 1:

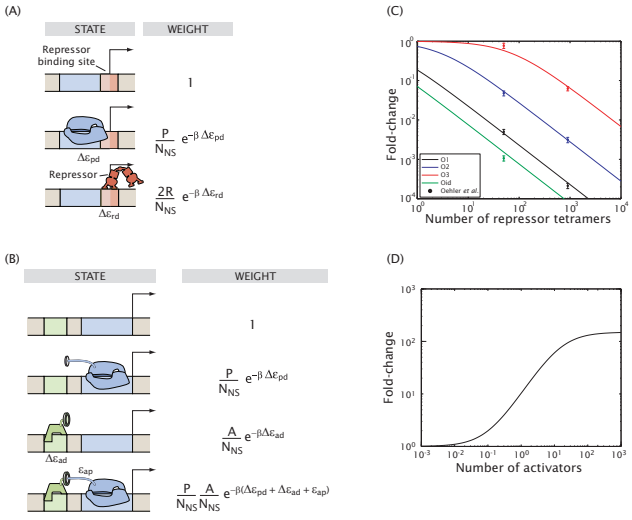
(A)
STATE
WEIGHT
Repressor binding site
1
$\frac{P}{N_{NS}} e^{-\beta \Delta\varepsilon_{pd}}$
$\Delta\varepsilon_{pd}$
Repressor
$\frac{2R}{N_{NS}} e^{-\beta \Delta\varepsilon_{rd}}$
$\Delta\varepsilon_{rd}$
(B)
STATE
WEIGHT
1
$\frac{P}{N_{NS}} e^{-\beta \Delta\varepsilon_{pd}}$
$\frac{A}{N_{NS}} e^{-\beta\Delta\varepsilon_{ad}}$
$\Delta\varepsilon_{ad}$
ε_{ap}
$\frac{P}{N_{NS}}\frac{A}{N_{NS}} e^{-\beta(\Delta\varepsilon_{pd} + \Delta\varepsilon_{ad} + \varepsilon_{ap})}$
(C)
Fold-change
O1
O2
O3
Old
Oehler et al.
Number of repressor tetramers
(D)
Fold-change
Number of activators

Figure 2:

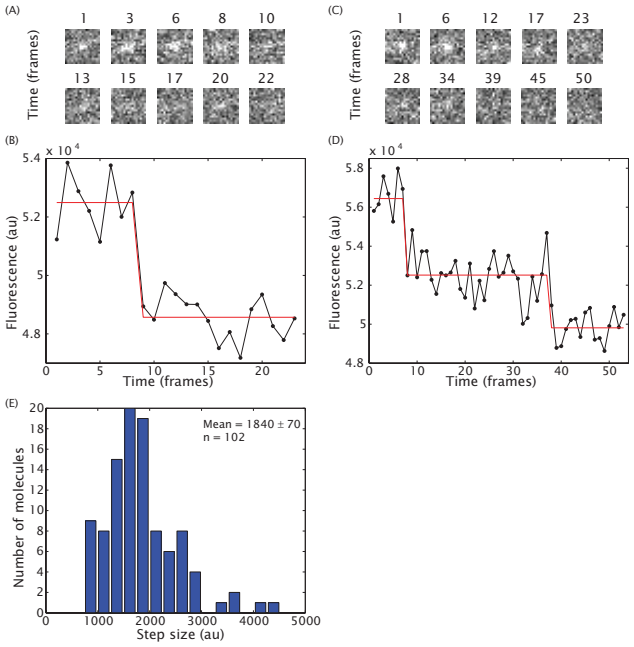
(A)
1 3 6 8 10
13 15 17 20 22
Time (frames)
(C)
1 6 12 17 23
28 34 39 45 50
Time (frames)
(B)
x 10^4
Fluorescence (au)
Time (frames)
(D)
x 10^4
Fluorescence (au)
Time (frames)
(E)
Mean = 1840 ± 70
n = 102
Number of molecules
Step size (au)

Figure 3:

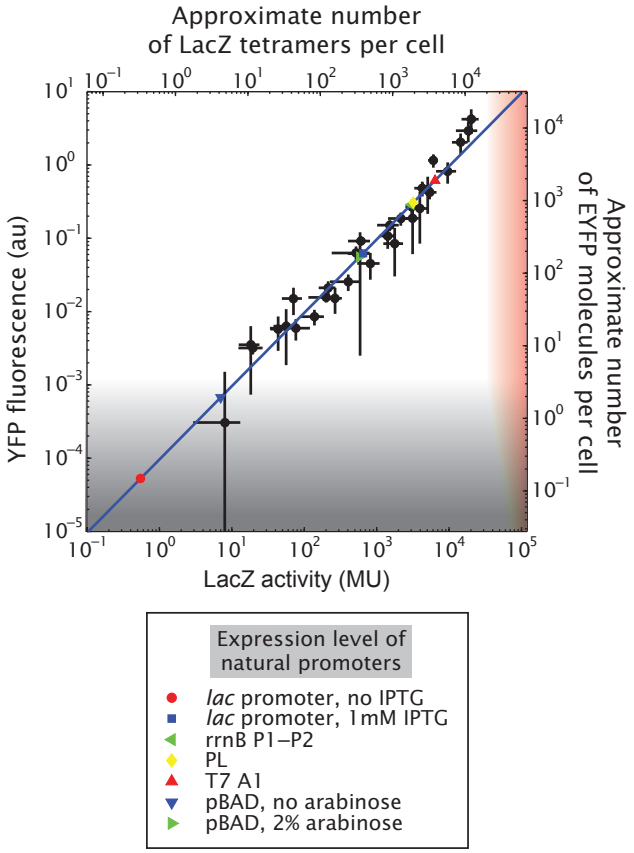
Approximate number
of LacZ tetramers per cell
YFP fluorescence (au)
LacZ activity (MU)
Approximate number
of EYFP molecules per cell
Expression level of
natural promoters
lac promoter, no IPTG
lac promoter, 1mM IPTG
rrnB P1–P2
PL
T7 A1
pBAD, no arabinose
pBAD, 2% arabinose

Figure 4:

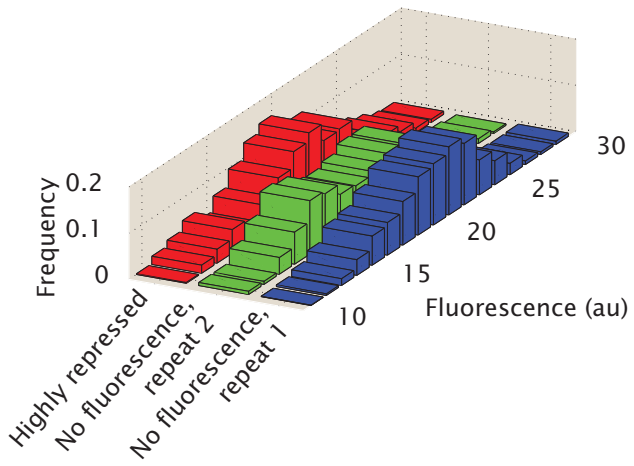
Frequency
0.2
0.1
0
10
15
20
25
30
Fluorescence (au)
Highly repressed
No fluorescence, repeat 2
No fluorescence, repeat 1

Figure 5:

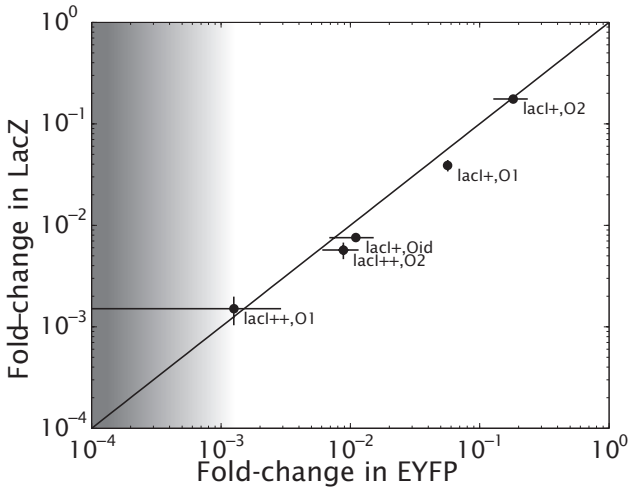
Fold-change in LacZ
Fold-change in EYFP
lacI+,O2
lacI+,O1
lacI+,Oid
lacI++,O2
lacI++,O1

Figure 6: