Supporting Online Material for

Probing Gene Expression in Live Cells, One Protein Molecule at a Time

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Supporting Online Material

Materials and Methods

E. coli strains construction

E. coli K-12 strain SX4 expressing Tsr-Venus was constructed by using the previously published λRED recombination system (1). Specifically, the chimeric *tsr-venus* gene, in which the coding sequence of *tsr* gene was fused to the N-terminus of *venus*, together with a kanamycin drug resistance marker was incorporated into the *lac*Z locus, replacing the native *lac*Z gene from the start codon ATG, and leaving intact the *lac* promoter and associated gene expression regulation elements, such as the CAP protein binding site, *lac* promoter, *lac* operator (O_1 and O_3) and *lac*Z ribosome binding site. The *lac* operator O_2 site is removed because it is within the coding sequence of *lac*Z gene. However, this change should only result in a small increase (less than five fold (2)) in gene expression level comparing with the native *lac* genes. The host *E. coli* K-12 strain BW25993 (CGSC) has a genotype of (*lac*Ip4000 *hsd*R514 DE(*ara*BAD)567 DE(*rha*BAD)568 rph-1). A similarly constructed control strain SX25 (expressing wild type Venus under the same promoter as in strain SX4) was provided by P. Choi, and the Venus gene was a kind gift from Dr. Miyawaki (3). The sequences of the above strains are available upon request.

Growth conditions and media

E. coli cells are grown in M9 glucose media supplemented with amino acids (Gibco), vitamin (GibCo) and appropriate antibiotics (kanamycin: 35 μg/ml, ampicillin:

50 μg/ml) overnight. The overnight culture was re-inoculated (1:200) into fresh M9 media with same supplements and antibiotics and grown until OD_{600} reaches 0.2. M9-based media is used instead of other rich medias (e.g. LB media) in order to maintain a well-defined cell culture condition and to achieve low cellular autofluorescence of *E. coli* cells.

For microscope experiments, cells were then washed twice in fresh media and resuspended in 20 folds dilution. A half micro-liter of the cell culture was spotted on top of an agarose gel pad made of 3% low melting temperature agarose in M9 media. A cleaned #1 coverslip was then placed on top of the agarose gel-pad to sandwich the cells in between. The sample chamber was then placed in a temperature-regulated sealed microscopy sample chamber (Bioptechs, FCS2) and was maintained at 37°C during the experiments.

The same culturing protocol was used for biochemical assays described in this paper, unless otherwise stated.

Time-lapse microscopy for detecting real-time gene expression

Fluorescence images of *E. coli* cells were taken with an epi-fluorescence microscope (Olympus IX71) with wide-field illumination of circularly polarized light from a 514-nm laser (Coherent, Innova Sabre) at a power density of 0.3 kW/cm^2. In order to monitor gene expression in real time, fluorescence images were taken every 3 min via a 60x microscope objective (NA=1.45, Olympus). To track the dividing cells, differential interference contrast (DIC) images are also taken and used for autofocusing at the cells. The fluorescence signal was collected with a cooled CCD camera (Roper Scientific,

VersArray1024). The image collection was controlled by the MetaMorph® (Universal Imaging). After each acquisition time (100 ms), Venus fluorophores are photobleached by 1100-ms laser exposure of 0.3 kW/cm^2.

Fluorescence images were further processed by thresholding to isolate the diffraction-limited spots of fluorescent fusion protein molecules inside cells. The intensity of a fluorescence spot is determined by integrating over 9 pixels around the intensity maximum, which is usually consistent with the single-molecule intensity in vitro (Fig. S2). Occasionally two or more fluorescent protein molecules cannot be resolved due to the limited spatial resolution, in which case the integrated intensity over the averaged single molecule intensity is used to determine the number of molecules.

Single molecule imaging of purified Venus molecules *in vitro*

We cloned the *venus* gene into expression vector pBAD202/D/TOPO (Invitrogen) and over-expressed in *E. coli* strain Top10 (Invitrogen). The protein was purified once with poly-His affinity column (ProBond™, Invitrogen) according to manufacturer's instructions. The purified protein was then biotinylated with succinimidyl D-biotin (Molecular probes), and immobilized on silanized glass surface coated with streptavidin (4). Single molecule images of the purified fluorescent protein were acquired at 100 ms exposure time with 514 nm laser excitation at 0.3 kW/cm^2 power density.

Synthesis of *tsr-venus* mRNA with i*n vitro* transcription for calibration of real-time RT-PCR assay

Linear DNA carrying the sequence of T7 promoter and *tsr-venus* fusion gene was generated from PCR reactions using a plasmid (pVS152/tsr) carrying *tsr-venus* fusion gene sequence as a template. Subsequently, *tsr-venus* mRNA was synthesized through *In vitro* transcription using the linear DNA as a template using MEGAscriptT7 kit (Ambion) according to manufacturer's instructions. The resulting mRNA was purified once with MEGAclear kit (Ambion) and quantified with absorption spectrometry at 260 nm. The purity of the RNA is verified with agarose gel electrophoresis.

Quantifying mRNA expression level with real-time RT-PCR

SX4 cells were cultured in M9 media as described above until OD600 reached 0.1 – 0.2. Small amount of cell culture (40-100 μl) were directly added to heated (95°C) chaotropic lysis buffer containing guanidine salt to release RNA molecules. Total RNA was purified with RNeasy RNA purification kit (Qiagen) according to manufacturer's instruction. The exact cell density of the culture was measured with a cell counter (Hausser Scientific) immediately, and verified by counting colonies generated on a non-selective LB plate after serial dilutions. After DNA contamination had been cleaned up using TurboDNA-free kit (Ambion), extracted *tsr-venus* mRNA was quantified with real time RT-PCR on ABI 7500Fast real-time PCR system using 5'CACATGAAGCAGCACGACTT3', 5'CGTCGTCCTTGAAGAAGATGGT3' as primers, and (6-FAM)CATGCCCGAAGGCTAC(MGB) as a gene specific Taqman probe. For each RNA preparation, four RT-PCR reactions were run in parallel, as well as no-RT control to ensure that the signal was due to RNA molecules. Negative control was carried out with BW25993 *E. coli* cells. Calibration is performed using *in vitro*

transcription generated mRNA. Specifically, we mixed BW25993 cells with known amount of *tsr-venus* mRNA, which was obtained from *in vitro* transcription, at the amount of $3.0x10^4$, $8.0x10^4$, $2.0x10^5$, $6.0x10^5$, $2.0x10^6$ and $6.0x10^6$ molecules respectively. The total RNA of the mixture was purified and measured by real-time RT-PCR, according to the same procedure described above. The resulting calibration curve was shown in Fig. S5. Multiple independent measurements on SX4 strain were performed and the results were summarized in Table S1.

Measuring cellular lifetime of *tsr-venus* mRNA with real-time RT-PCR

To determine the *tsr-venus* mRNA cellular lifetime, we used real-time RT-PCR to follow mRNA decay after pulse transcription induction. IPTG was first added to 1mM final concentration to SX4 cells at early log phase (OD_{600} ~ 0.3). After 5 minutes of induction with IPTG, transcription inihibitor Rifampicin at a final concentration of 350 μg/ml was added into the cell culture and aliquots of the culture were taken and frozen at different time points. Reverse transcription of the whole cell mRNAs was performed using the SuperScript TM III First-Strand Synthesis System from Invitrogen. A set of primers for amplifying *tsr-venus* cDNA in the PCR reaction (5'-GACGTAAACGGCCACAAGTT-3 and 5'-AAGTCGTGCTGCTTCATGTG-3') was designed for the detection of the *venus* coding region. Real time PCR was performed on MJ Opticon using SYBR green RT-PCR master mix (ABI). The data was plotted in Fig. S4. A single exponential fit of the decay curve reveals an mRNA degradation time constant $\tau_{mRNA} = 1.5 \pm 0.2$ min.

Protein expression quantification with Western blotting assay

Following cells' growth into the early log phase (OD_{600} ~ 0.3), IPTG was added to the cultures at a final concentration of 1 mM and the cells were allowed to further grow for five hours. 1 ml of cells was then harvested and re-suspended in 1X PBS. Cells' concentrations in the suspensions were determined by counting the cell numbers using a Petroff Hausser Counting Chamber (Hausser Scientific). Cells were lysed by the addition of a 3X SDS loading buffer (New England Biolab) with additional SDS (final concentration 4%) and incubated at 95°C for 10 minuets. Appropriate amounts of the cell whole lysates were loaded on a 10-well mini 4-15% gradient acrylamide Tris-HCl precast gels (BioRad). The recombinant GFP (BD Biosciences Clontech) with known concentration was used as a standard to calibrate the concentrations of the expressed Tsr-Venus fusion protein and wild type Venus protein in the whole cell lysates. The gels were run at 90 Volts for 120 minutes and transferred to PVDF membrane (Sigma) at 30 mA overnight using Protean 3 mini electrophoresis system fitted with mini Trans-Blot module (BioRad). The blots were hybridized using a Living Colors ® A.v.(JL-8) mouse monoclonal primary anitbody (BD Biosciences Clontech), subsequently hybridized with anti-mouse monoclonal IgG secondary antibody and reacted with CPS substrates (Sigma, ProteoQwest Chemiluminescent kit) according to manufacture's instructions. The signals corresponding to the protein bands were detected using Alpha Innotech Imager System, and the intensity of the Tsr-Venus and wild type Venus bands are quantified against that of the standard recombinant GFP using a home-made Matlab program.

Supporting Text

1. Western characterization of Tsr-Venus protein expression in *E. coli*

We compared the expression levels of Tsr-Venus (strain SX4) and unmodified Venus (strain SX25) under the control of *lac* promoter at induced level. Both strains have the reporter protein expressed under the control of *lac* promoter on chromosome. Cells were harvest at early log phase (OD_{600} ~ 0.3). The expressions of Venus proteins were measured with Western blot and normalized against cell numbers. The relative expression levels of the two strains were plotted in Fig. S1. Within experimental error range (six repeated measurements), the relative expression level of 1.3 ± 0.4 of the membrane targeted Tsr-Venus is similar to that of the wild type Venus of 1.0 ± 0.3, indicating little exogenous effect due to the *tsr* fusion.

We also measured the repressed expression level of Tsr-Venus (strain SX4) using quantitative Western Blotting assay as described above. In absence of an inducer, the average expression level of Tsr-Venus was quantified as 3.9 ± 2.8 copies of proteins per cell (15 repeated measurements). The number is consistent with the time-averaged and population-averaged expression levels of single cells measured under the microscope (see main text for detail). The agreement between the fluorescence measurements and Western suggests that a sufficiently high percentage of the fluorescent protein fold, incorporate into the membrane, and mature to fluoresce.

2. Quantification of *tsr-venus* mRNA expression level in repressed condition

In order to determine the average number of mRNA molecules produced in each expression burst, we carried out real-time RT-PCR experiments to quantify average

number of *tsr-venus* mRNA molecule in SX4 cells at repressed condition. Total RNA was extracted from a large number (~10^7) of SX4 cells and measured with real-time RT-PCR assay designed to detect *venus* coding sequence. The measurements were repeated seven times on three different days, and the result is shown in Table S1. The average number of *tsr-venus* mRNA at our culture condition is 36.9 ± 12.9 (at 95% confidence level) molecules per thousand cells. This result enables us to determine the average number of mRNA molecules produced in each expression burst according to $m = n_{mRNA}\tau_{cell} / (n_{burst}\tau_{mRNA})$, where n_{mRNA} is the average number of mRNA molecules per cell, τ_{cell} is the average cell division time (55 minutes), n_{burst} is the average number of expression bursts per cell division cycle (1.2), and τ_{mRNA} is the cellular lifetime of the *tsr-venus* mRNA (1.5 ± 0.2 min). The resulting *m* at our experimental condition is 1.14 ± 0.42. Furthermore, the geometric distribution in protein production (Fig. 4B) implies that each mRNA molecule has the probability of $1-\rho$ to produce no protein (n = 0). Therefore, the average number of "effective" (protein producing) mRNA per protein expression burst is $m \cdot \rho = 0.91 \pm 0.3$. This corroborates the one-to-one correspondence of mRNA and protein expression burst under the repressed condition.

3. Clustering of Tsr-Venus at cell poles

Tsr-Venus protein molecules expressed in *E. coli* cells cluster at cell poles as shown in Fig. S6, indicating that Tsr-Venus molecules follow the same localization pattern as Tsr, which is known to localize to cell pole. When the production of Tsr-Venus is induced with IPTG, the integrated fluorescence intensity increase by more than 300 folds, consistent with the expected increase of protein production level. These data

indicate that there is no self-quenching problem of the Venus chromophores due to the clustering of the protein molecules and the integrated fluorescence intensity of Tsr-Venus is an accurate measure of the gene expression level at high expression level. We also found no indication of toxicity at the elevated expression of Tsr-Venus (cell division rate slows down only slightly). This is not surprising considering that the endogenous Tsr protein is normally expressed at very high level in *E. coli* cells.

4. Temporal spread of the gene expression bursts

The average temporal width of the gene expression bursts is 7.0 ± 2.5 min, corresponding to the average spread of the stochastic arrival times of the fluorescent reporter proteins within a burst. The entire protein production process consists of transcription, translation, and post-translational assembly that includes folding, membrane incorporation, and fluorophore maturation. Assuming one mRNA molecule per gene expression burst, we consider the following three facts in assigning the rate-limiting step for protein production. First, in *E. coli*, transcription and translation are coupled and the translation rate is ~15 amino acids per second. Therefore, the synthesis of an 815-amino-acid fusion polypeptide is completed within 1 min. Second, the separation of two adjacent ribosomes on the mRNA is ~30 nm according to electron micrograph studies (5), implying that the separation between two consecutive polypeptides can be as short as ~3 s. Third, the *tsr-venus* mRNA lifetime is only ~1.5 min. Therefore we assign the 7-min burst width to the post-translational assembly. We tentatively assign the rate constant, κ, to the fluorophore maturation process, though we cannot experimentally rule out the other two possibilities. In principle, a faster maturing

construct would allow the kinetic measurements of protein folding and membrane incorporation.

5. Autocorrelation function of protein production time traces

We model the reporter protein (Tsr-Venus) production process inside single cells in continuous time as a train of random pulses, each presenting the event of producing one fluorescent protein. Thus a time trace of the protein production is the sum of a series of delta functions:

$$\chi(t) = \sum_i \delta(t - t_i), \tag{S1}$$

where t_i is the time of the ith event. The probability density of finding any two events separated by a delay time τ is:

$$G(\tau) = \lim_{N\to\infty} \frac{1}{N} \sum_{i,j} \delta(t_j - t_i - \tau), \tag{S2}$$

where N the total number of events. The autocorrelation function of this trajectory is closely related to $G(\tau)$:

$$\begin{aligned} C^{(2)}(\tau) &\equiv \lim_{T\to\infty} \frac{1}{T} \int_{-T/2}^{T/2} \chi(t)\chi(t+\tau)dt \\ &= \lim_{T\to\infty} \frac{1}{T} \sum_{i,j} \delta(t_j - t_i - \tau) \\ &= vG(\tau) \end{aligned} \tag{S3}$$

where v is the average event rate, and T is the total length of the time trace.

We now derive the autocorrelation function based on the following three assumptions. First, we assume the transcription initiation events are temporally

uncorrelated (i.e. a Poisson processes). Therefore, the waiting time distribution between *any* two such events is constant:

$$C_0(t) = s \tag{S4}$$

where s is the rate of mRNA production. Second, the number of protein molecules n translated from one mRNA follows a geometric distribution:

$$P_n(n) = \rho^n(1-\rho)\,. \tag{S5}$$

where ρ is defined in main text Eq. 1. It follows that the average number of proteins produced from one mRNA $\langle P_n \rangle = \rho/(1-\rho)$ and the average protein production rate $v = s\rho/(1-\rho)$. Third, we assume there is one rate-limiting step in the post-tranlational assembly of the protein, and the transcription/translation times for fluorescent proteins are short. Therefore, the probability density for the protein assembly time (the waiting time between the transcription initiation and the formation of a fluorescent protein) follows the distribution of a single exponential decay:

$$p_G(t) = \begin{cases} \kappa \exp(-\kappa t) & t \geq 0 \\ 0 & t < 0 \end{cases}, \tag{S6}$$

where κ is the rate constant of post-translational assembly of the fluorescent protein.

We will now calculate $G(\tau)$ in two parts. First we consider the situation in which two proteins molecules, produced at time t_i and t_j, are translated from different mRNA molecules. Because the mRNA productions are uncorrelated events, we expected synthesis of these two protein molecules also to be uncorrelated, i.e. the probability density of the waiting time is constant. This can be proven as the following:

$$\tau = t_j - t_i = t_m - t_1 + t_2$$
$$G_1(\tau) = \int_0^\infty dt_1 \int_{-\infty}^\infty dt_m [p_G(t_1) C_0(t_m) \sum_n P_n(n) n \cdot p_G(t_2)] \quad \text{(S7)}$$
$$= \frac{s\rho}{1-\rho} = v$$

where t_1 is the ith protein molecule's assembly time, t_2 is jth protein molecule's assembly time, and t_m is the delay between the two transcription events corresponding to ith protein and the jth protein respectively.

Secondly we consider the case in which two protein molecules are translated from the same mRNA molecule. In this case only t_1, t_2 need to be considered:

$$G_2(\tau) = \frac{\sum_n P_n(n) \cdot n \cdot (n-1)}{\sum_n P_n(n) \cdot n} \int_0^\infty dt_1 [p_G(t_1) p_G(\tau + t_1)] \quad \text{(S8)}$$
$$= \frac{\kappa\rho}{1-\rho} \exp(-\kappa\tau)$$

The total probability density is the sum of G_1 and G_2. Therefore,

$$C^{(2)}(\tau)$$
$$= vG(\tau) = v[G_1(\tau) + G_2(\tau)] \quad \text{(S9)}$$
$$= \left(\frac{s\rho}{1-\rho}\right)^2 [1 + \frac{\kappa}{s} \exp(-\kappa\tau)]$$

which shows that the autocorrelation function is a single exponential decay with a spike at τ=0 time, an offset at $\tau \to \infty$, and the decay time constant being the average assembly time of fluorescent protein.

In practice, the experimentally measured time trace has a finite time bin Δ (3 min) in our experiment, and $C^{(2)}(\tau)$ is a discretized version Eq. S9. The value of the time trace at the time grid t_s is $\hat{\chi}(t_s) = \sum_i \hat{u}(t_s - t_i)$, where $\hat{u}$ is defined as $\hat{u}(t_s - t_i) = 1$ for

$-\frac{1}{2}\Delta \le t_s - t_i < \frac{1}{2}\Delta$ and $\hat{u}(t_s - t_i) = 0$ otherwise. The autocorrelation function obtained from the time trace is

$$\hat{C}^{(2)}(\tau) = < \hat{\chi}(0)\hat{\chi}(\tau) > , \tag{S10}$$

which is related to $C^{(2)}(\tau)$:

$$\begin{aligned} \hat{C}^{(2)}(\tau) &= \int_{\tau-\Delta}^{\tau} C^{(2)}(t)\,\tfrac{t-\tau+\Delta}{\Delta}\,dt + \int_{\tau_s}^{\tau+\Delta} C^{(2)}(t)\,\tfrac{\tau-t+\Delta}{\Delta}\,dt \\ &= \frac{2[1-\cosh(\kappa\Delta)]}{(\kappa\Delta)^2}\cdot C^{(2)}(\tau)\,. \end{aligned} \tag{S11}$$

Therefore when $\kappa\Delta \le 1$, as is the case in our experiment, $\hat{C}^{(2)}(\tau) \approx C^{(2)}(\tau)$. Eq. S11 does not consider the cell cycle dependence variation, which occurs at a longer time scale than κ^{-1}.

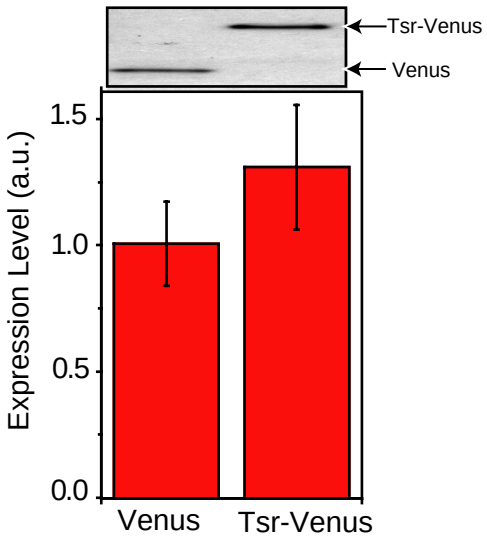

Fig. S1. Comparison of the induced expression levels of Venus (strain SX25) and Tsr-Venus (strain SX4) using a quantitative Western assay. The top panel shows the Western blot gel picture. The relative amounts of expression levels were plotted in the bottom panel for Venus and Tsr-Venus respectively. Each measurement was repeated six times. The error bar depicts the standard deviation of the measurements. The similar expression levels indicate little exogenous effect due to the addition of the *tsr* sequence.

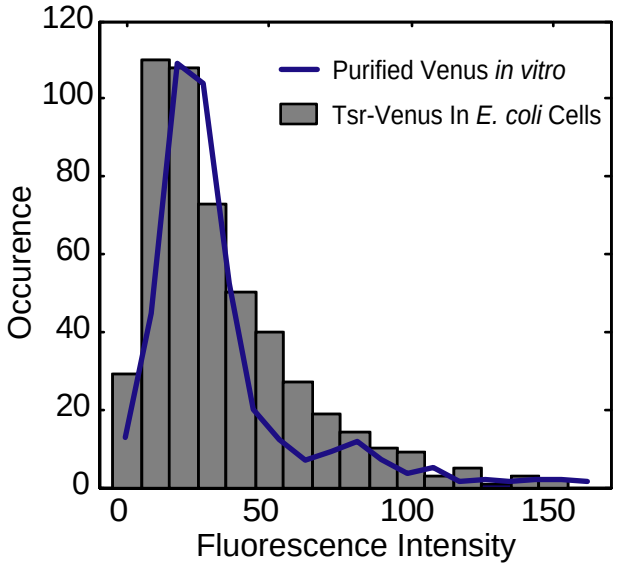

Fig. S2. Distribution of fluorescence intensity of single Venus molecules. The gray bars represent fluorescence intensities of individual fluorescence spots inside *E. coli* cells at low expression levels of Tsr-Venus (strain SX4). The solid curve represents the distribution for purified Venus molecules immobilized on glass surface.

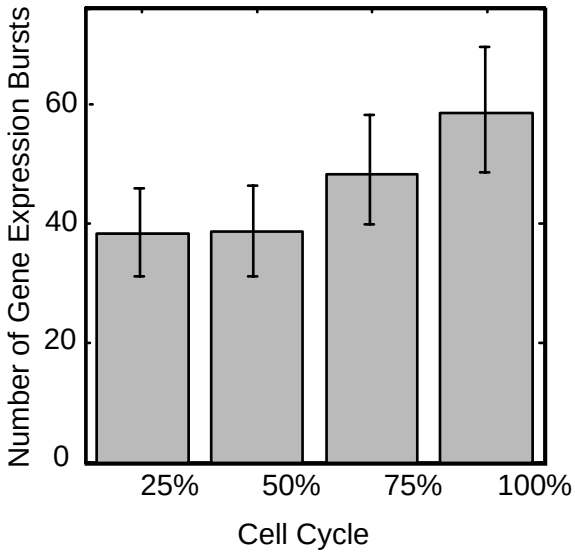

Fig. S3. Cell cycle dependence of the gene expression rate for SX4 at the repressed condition. The division cycle of each cell was divided into four time windows of equal length. The number of gene expression bursts in each window was counted for >200 cells. The standard deviation is estimated by dividing the cells randomly into four groups and counts each group separately. The result shows that more gene expression bursts are observed at the later stage of the cell cycle.

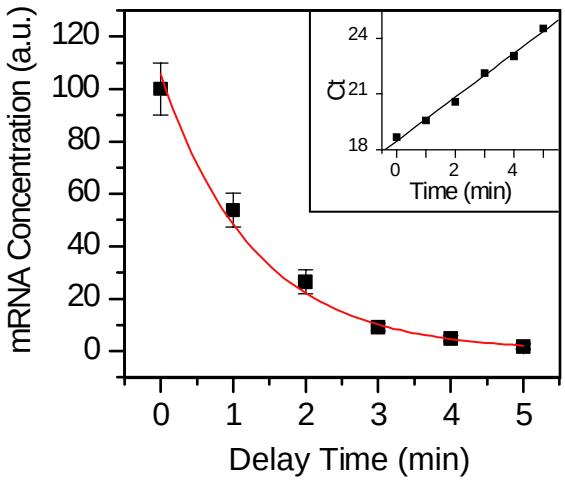

Fig. S4. Cellular lifetime of *tsr-venus* mRNA. After a short period of induction, the mRNA production is inhibited and the amount of *tsr-venus* mRNA is measured using real time PCR. The measurement is repeated six times. The error bar depicts the standard deviation of the measurements. The decay of the *tsr-venus* mRNA (squares) is fitted with a single exponential function (solid curve), which yields an mRNA degradation time constant of 1.5 ± 0.2 min. The inset shows the threshold cycle number (Ct) measured in the real-time PCR versus the time delay. The straight line is a linear fit of the data.

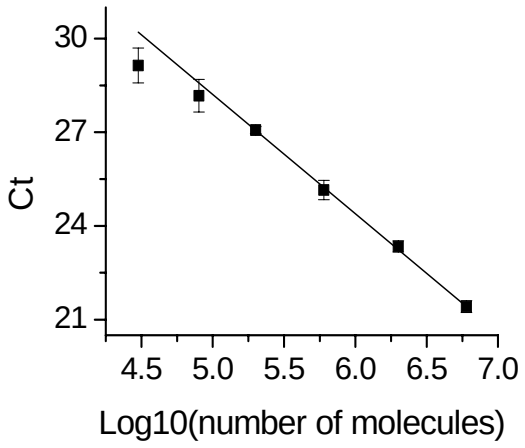

Fig. S5. Calibration for the real-time RT-PCR quantification of *tsr-venus* mRNA. The total RNA is extracted from ~10^7 BW25993 cells mixed with known amount of *in vitro* synthesized *tsr-venus* mRNA. The amount of *tsr-venus* mRNA is quantified with real-time RT-PCR. The average threshold cycle number (Ct) from four independent extractions was plotted against the amount of *tsr-venus* mRNA added. The error bar depicts the standard deviation of Ct. The straight line is a variance weighted linear fit through all the measurements, except the data point at lowest amount of mRNA ($3x10^4$ molecules), where the measured Ct deviate obviously from the linear region, due to presumably the non-specific signal background from the BW25993 cells. RNA extracted from BW25993 cells without adding any *tsr-venus* mRNA gives a Ct of 30.

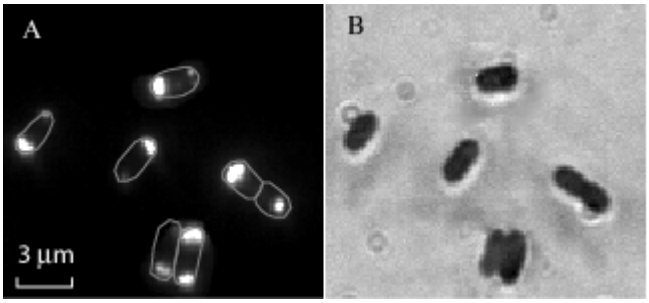

Fig. S6. Fluorescence (A) and phase-contrast (B) images of SX4 cells with induced Tsr-Venus expression. IPTG was at to SX4 culture at early log phase to induce the expression of Tsr-Venus. Cells were grown for two more hours before imaged on fluorescence microscopy with 514 nm laser excitation to detect the presence of Tsr-Venus fluorescence. The cell boundaries are outlined in the fluorescence image according to the phase-contrast image. All cells show prominent clustering of Tsr-Venus protein molecules at the cell pole. The integrated intensity of the fluorescence signal corresponds to the expected increase of protein production (>300 folds increase compared to SX4 cells culture without IPTG).

Table S1. Quantification of *tsr-venus* mRNA in SX4 with real-time RT-PCR

	Ct	Number of mRNA molecules	Number of cells	mRNA molecules per 1000 cells
1	24.15 ± 0.12	1154770	1.88×10^7	61.42
2	25.75 ± 0.16	439314	1.88×10^7	23.37
3	26.82 ± 0.16	230963	1.20×10^7	19.25
4	25.69 ± 0.09	454579	1.20×10^7	37.88
5	25.95 ± 0.01	390204	1.20×10^7	32.52
6	25.16 ± 0.07	626939	1.53×10^7	41.11
7	25.10 ± 0.05	648723	1.53×10^7	42.54
Average				36.9
95% confidence interval				36.9 ± 12.9

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Supporting Movie S1 & S2:

Time-lapse movies (DIC/Fluorescence overlay) of SX4 cells under the repressing condition. Single fluorescent protein molecules are spontaneously generated, and clustered in gene expression bursts. The images were collected every 3 min with 100 ms acquisition time followed by 1100 ms photobleaching time. The total elapse time is 210 min for the first movie and 170 min for the second.