**Supplementary File 2: In vivo kinetics of transcription initiation of the lar promoter in Escherichia coli. Evidence for a sequential mechanism with two rate-limiting steps.**

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I. qPCR analysis of the target RNA:

Gene expression was induced as described in the material and methods section in the main document. Total RNA was isolated using RNeasy kit (Qiagen) according to the manufacturers’ instructions. DNaseI treatment was performed before cDNA synthesis. Maxima® First Strand cDNA Synthesis Kit was used to make cDNA (Fermentas). Primers for mRFP1, 16S rRNA were used to amplify the desired length. Real time PCR experiment was performed using Maxima™ SYBR Green qPCR Master Mix in a Biorad MiniOpticon Real time PCR system. The following thermal cycling protocol was used: 35 cycles of 94°C for 15 s, 54°C for 30 s, and 72°C for 30 s for each cDNA replicate. RT–qPCR reactions were performed by using primers (mRFP1-Fw: 5' TAC GAC GCC GAG GTC AAG 3'; mRFP1-Rv: 5' TTG TGG GAG GTG ATG TCC A 3’) for the target gene and primers for housekeeping genes (16S rRNA-Fw: 5'CGT CAG CTC GTG TTG TGA A 3'; 16S rRNA-Rv: 5' GGA CCG CTG GCA ACA AAG 3’) as an internal reference. Replicate samples were used to quantify the gene expression in the final volume of 20μL reaction. Two independent sets of experiments were performed with primer efficiencies of these reactions not less than 90%. CFX Manager™ Software was used to obtain the amplified gene expression pattern for the target and 16S rRNA housekeeping gene and then the Livak method (for reference see main document) was used to confirm the relative gene expression changes.
Results are shown in supplementary figure 1, and the change in RNA relative expression level with induction (from weak to medium) is in accordance with the changes measured in live cells under confocal microscopy (reported in the main document).

**Supplementary figure 1: mRFP1 gene expression analysis:** The relative changes in mean mRNA numbers with induction strength were measured with quantitative real time PCR. Target RNA was induced with weak and medium concentrations of the inducers. We observe that mRFP1 gene expression in the medium induction case is 1.6 times that of the weak induction case, as calculated by the Livak method. Error bars indicate the statistical significance between two independent measures of real time experiments.
II. Image analysis and cell segmentation. Extraction of the number of RNAs in each cell at each moment.

Once the images of the cells are acquired by confocal microscopy (supplementary figure 2), we analyze them in a semi-automated fashion to detect and count the target RNA. This process starts by segmenting the cells as well as the RNA spots within. From there, the distribution of spot intensities from cells in each image can be obtained.

Supplementary figure 2: Examples of original and segmented images of cells and spots within: A-C.

Examples of images taken by confocal microscope of MS2-GFP-tagged RNA molecules in *E. coli* cells. Unprocessed image (left) and the corresponding segmented image (right) showing the detected cells in grey and the spots in white.
The number of tagged transcripts in a cell can be estimated by dividing a spot’s intensity by the intensity of the first peak in the histogram of spots intensities (supplementary figure 3). Relevantly, at very low induction levels, only one peak is detected, and it corresponds to the intensity of a single tagged RNA molecule (see below, as well as the main document and ref. [12] within). According to this method, here referred to as the “slicing approach”, the estimated number of RNA molecules per spot is equal to its total fluorescence, normalized by the intensity of a single tagged mRNA molecule and rounded to the nearest integer. The intensity of one RNA molecule equals the intensity of the first peak, as calculated from the tagged mRNA molecules (supplementary figure 2).

Supplementary figure 3: Counting of mRNA in cells: Distribution of number of spots and their intensity in a cell population. The estimated number of RNA molecules per spot is equal to its total fluorescence normalized by the intensity of a single tagged mRNA molecule (intensity of the first peak).

For a time series measurement, the process of segmentation is made for each individual image, independently. Then, the overall distribution of spot intensity is generated, obtained from all cells at each time point. From that, the number of RNA molecules in each cell, at each time point, can be obtained. By counting the number of RNAs in each cell at each moment, it is
possible to determine when a new RNA appears and, thus, the time between the appearance of consecutive RNA molecules in individual cells. From that, we can generate distributions of intervals between consecutive transcription events for each cell, in multiple cells subject to the same level of induction of the target gene.

**IV. Time intervals between production events assuming an ON-OFF mechanism of RNA production.**

In the paper ref. [33] in the main document, a two rate limiting step model is assumed to explain the observed measurements of cell to cell diversity in RNA numbers as calculated by the Fano factor of RNA numbers extracted from measurements by single-molecule fluorescence in situ hybridization (FISH). The model of RNA transcription and degradation can be represented by the following set of reactions:

\[
\begin{align*}
\text{Off} & \xrightleftharpoons[k_{\text{on}}]{k_{\text{off}}} \text{On} \\
\text{On} & \rightarrow \text{mRNA} \\
\text{mRNA} & \rightarrow \emptyset \quad (1)
\end{align*}
\]

In reaction (1), ‘Off’ is the promoter in the OFF state, ‘On’ is the promoter in the ON state, mRNA is a complete RNA molecule, and the symbol \( \emptyset \), resultant from the last reaction, represents the degradation of the RNA. The RNA level is determined by the various rate constants, namely, by \( k_{\text{on}} \), the rate of switching to the ON state; by \( k_{\text{off}} \), which sets the rate of switching from the ON to the OFF state; and by \( k_{\text{TX}} \), which is the rate of producing RNA molecules while in the ON state. The last reaction is a first order degradation reaction for the mRNA, with rate \( k_d \). Changes in the dynamics of RNA numbers occur if one modifies any of the kinetic parameters controlling RNA production and degradation.
It is possible to show that, for any value of the kinetic rates of RNA production, this model always produces a coefficient of variation of time intervals between consecutive RNA molecules (standard deviation over the mean) that is greater than 1, for a population of cells at any given moment. Let $T_R$ be the distribution of intervals between the productions of two consecutive RNA molecules. The distribution of $T_R$ is determined by the three production rates above. It is possible to show that the mean and variance of this distribution are given by:

$$E[T_R] = \frac{1}{k_{tx}} \left( 1 + \frac{k_{off}}{k_{on}} \right)$$  \hspace{1cm} (2)

$$Var[T_R] = \frac{1}{k_{tx}^2} \left( 1 + \left( 2 + 2 \frac{k_{tx}}{k_{on}} + \frac{k_{off}}{k_{on}} \right) \frac{k_{off}}{k_{on}} \right)$$  \hspace{1cm} (3)

From (2) and (3), one can calculate the coefficient of variation. By evaluating the resulting expression, it is possible to see that it is always be greater than 1.

The distribution of $T_R$ itself resembles an exponential distribution with a heavy tail (thus it differs significantly from our measured distributions shown in the main document). Since this distribution has its mode at 0 and many long intervals where no transcription occurs, the difference between this model and the one presented in the main document are apparent. Sampled distributions of this model are shown in supplementary figure 4.
Supplementary figure 4: Probability distribution of intervals between consecutive RNA molecules for varying $k_{\text{off}}$. These distributions are derived from the model described by supplementary equation (1) for three values of $k_{\text{off}}$. Values of $k_{\text{on}}$ and $k_{\text{off}}$ are set to 0.2 and 0.004 s$^{-1}$, respectively (as in ref. [33] in main document). Binning is set to 10.

V. Measurements under full induction:

We were unable to observe full induction in time series measurements. To observe full induction, cells need to be kept in liquid and optimal growth condition just prior to measurement. These measurements thus consist of images of cells taken 1 hour after full induction (1 mM IPTG and 6.7 mM Arabinose). The cells are kept in liquid culture until the moment of measurement. We measured 4.04 RNA/cell from a population of 186 cells. Two replicas obtained in independent experiments confirmed this result. These results agree with previous reports (referenced in the main document). An example image of cells of such populations is shown in Supplementary Fig. 5.
Supplementary figure 5: Detection of mRNA in cells of a population 1 h following induction. The cells are subject to full induction and kept in liquid culture until the moment of measurement. Most spots correspond to more than a single RNA molecule and, thus, the number of RNA molecules per cell can only be counted following the slicing approach.