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Electronic supplementary information

Single-cell kinetics of the Repressilator when inserted into a single-copy plasmid

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Supplementary Methods

Media and chemicals

We used Lysogeny Broth (LB) and minimal nutrient (M63) media with the following components (i) and (ii), respectively: (i) 10g/L of Tryptone (Sigma Aldrich, USA), 5g/L of yeast extract (LabM, UK) and 10g/L of NaCl (LabM, UK); (ii) 2 mM $MgSO_4.7H_2O$ (Sigma-Aldrich, USA), 7.6 mM $(NH_4)_2SO_4$ (Sigma Life Science, USA), 30 µM $FeSO_4 \cdot 7H_2O$ (Sigma Life Science, USA), 1 mM EDTA (Sigma Life Science, USA), 60 mM KH_2PO_4 (Sigma Life Science, USA) pH 6.8 with Glycerol 0.5% (Sigma Life Science, USA) and Casaminoacids 0.1% (Fluka Analytical, USA).

Isopropyl β-D-1-thiogalactopyranoside (IPTG) was used for studying the effects of external perturbations on the Repressilator. All antibiotics used for SCR and LCR strain culturing were purchased from Sigma-Aldrich (USA): (i) 35 mg/mL kanamycin and 35 mg/mL chloramphenicol; (ii) 35 mg/mL kanamycin and 20 µg/mL Ampicillin. Agarose (Sigma Life Science, USA) was used for the microscopic slide gel preparation.

Bacterial strains and single-copy repressilator plasmid construction and validation

Cells of *E. coli lac*$^-$ strain MC 4100 with the repressilator (pZS1-lTlrLLtCL), here denoted by low-copy repressilator (LCR), and the reporter plasmid (pZE21-GFPaav) were generously provided by M. Elowitz, Princeton University, NJ, USA. Cloning and measurements were performed on this strain.

To construct the single-copy F-plasmid repressilator (SCR) system pBAC2-lTlrLLtCL, we amplified the functional repressilator cassette from the original plasmid (*de novo*

SmaI restriction sites were added to the end of the cassette during this procedure). The primers used were:

1-Rep.SmaI-Fw: 5' CCCGGGTCGAGAATTGTGAGCG 3'
2-Rep.SmaI-Rev: 5' CCCGGGTCAAGCTGCTAAAGCGTAG 3'

The vector, pTB-BAC2 F-plasmid, containing the origin of replication and Chloramphenicol resistance gene, was amplified using PCR, also amplified with *SmaI* restriction enzyme sites, using the following primers:

3-Sc.ori.Cam-SmaI-Fw: 5' CCCGGGTTCGAACGCGTATGCATGAG 3'
4-Sc.ori.Cam-SmaI-Rev: 5' CCCGGGTTAGGGCCGTCGACCAA 3'

The amplified sequences of the repressilator and pTB-BAC2 vector were digested using SmaI and then ligated. The plasmid was then transferred into *lacI*$^-$ *E. coli* MC4100 containing the reporter plasmid.

We validated the SCR construction by performing gel electrophoresis (Fig. S4, for the construction; Fig. S5 for the final product) to confirm the presence of the Repressilator circuit in the single-copy plasmid.

Sequencing the plasmid for confirmation

A fraction (covering the vector and insert) of the new plasmid was amplified using PCR, from the Chloramphenicol resistance gene in the vector to the tetR region of the Repressilator, and sequenced using appropriate primers. The primers used for the amplification were:

5-CmR-1-F: 5' CCGCTGGCGATTCAGGTTC 3'
6-tetR-3-R: 5' AGCAAAGCCCGCTTATTTTTTACATG 3'

The alignment of the sequence obtained from sequencing against the expected original Repressilator sequence[2] using NCBI BLAST (S. F. Altschul et al., J. Mol. Biol. 1990, 215: 403-410) is shown in Fig. S6B. Further, the complete sequence of the single-copy repressilator plasmid is shown in Fig. S6C.

qPCR verification of RNA expression

qPCR was used to further validate the presence of each gene in the SCR plasmid in the host cell. For that, *lacI*$^-$ *E. coli* MC 4100 cells, containing the SCR with the reporter system, were grown following the culturing protocols described in the methods section of the manuscript. After 10 hours of culturing in 5 mL liquid M63 medium at shaking 250 rpm, one sample was taken and rifampicin was immediately added to prevent further transcription. RNA protect reagent was used to fix the cells before their enzymatic lysis with Tris-EDTA lysozyme buffer (pH 8.3). The RNA was isolated from cells using RNeasy mini-kit (Qiagen) following manufacturer's instructions. Total of 1 µg of RNA was used as a starting material. To ensure purity of the RNA, the RNA samples were treated with DNase free of RNase to remove residual DNA. Next, the RNA was reverse transcribed into cDNA using iSCRIPT reverse transcription super mix (Biorad). Finally, qPCR was performed using Power SYBR-green master mix (Life Technologies) with primers for the amplification of the target and the reference genes at a concentration of 200 nM. Reactions were carried out in 20 µL triplicates with 500 nM per primer. The following primers were used for quantification:

-For *lacI* gene:
7-lacI.pro-Fw: 5' GTGGTGTCGATGGTAGAACG 3'
8-lacI.pro-Rev: 5' CTGTTGATGGGTGTCTGGTC 3'

- For *tetR* gene:
9-tetR.pro-Fw: 5' CGCTGTGGGGCATTTTAC 3'
10-tetR.pro-Rev: 5' AAGAAGGCTGGCTCTGCAC 3'

- For *cI* gene:
11-cI.pro-Fw: 5' GATGCGGAGAGATGGGTAAG 3'
12-cI.pro-Rev: 5' ACTCATCACCCCCAAGTCTG 3'

The length of the amplicons was kept at 90 bp. The sequences of the primers of the reference gene 16S rRNA (EcoCyc Accession Number: EG30090) were obtained from Thermo Scientific:

13-Fw: 5' CGTCAGCTCGTGTTGTGAA 3'
14-Rev: 5' GGACCGCTGGCAACAAAG 3'

The level of each target gene was normalized with the level of the 16S rRNA for all samples. The PCR cycling protocol used was 94 °C for 15 s, 51 °C for 30 s, and 72 °C for 30 s, up to 39 cycles. We used no-RT enzyme and no-Template as controls. The C_q values were obtained from the CFX Manager™ Software and the fold change of the genes were analysed using the Livak method (K.J. Livak, and T.D. Schmittgen, Methods, 2001, 25, 402-408).

For the SCR, we obtained the following cycle threshold (C_t) values: 18.7 (lacI), 17.3 (tetR), and 21.6 (cI). Meanwhile, in the no-Template control these numbers equalled 26.6, 33.2, and 31.4, respectively. These result in fold changes no smaller than 200, which indicates that the RNAs are being expressed in the SCR.

Stochastic model of the Repressilator and of coupled Repressilators

We implemented stochastic models of the LCR and of the SCR based on a model proposed by Zhu and others[11]. The models are implemented using the stochastic simulation algorithm, which is a Monte Carlo simulation of the stochastic chemical kinetics governed by the chemical master equation. Gene expression is modelled by the following reactions:

$$\varnothing \xrightarrow{N_i \cdot k_i} P_i \qquad k_i = \frac{a_i}{1 + \left(\frac{P_{\sigma(i)}}{H_i} \right)^{b_i}}$$

$$P_i \xrightarrow{d_i} \varnothing$$

where the first reaction represents the production of the proteins P_i (for $i = 1,2,3,4$), and the second their degradation. Here, N_i represents the copy number of the gene, k_i is the effective rate of protein production for a single gene (accounting for e.g. transcription and translation rates and the messenger RNA degradation of that gene), and d_i^{-1} is the protein lifetime. In our model P_1, P_2, P_3 and P_4 correspond to TetR, λ cI, LacI and the GFP reporter, respectively, and

the repressor indices are $\sigma(1)=3$, $\sigma(2)=1$, $\sigma(3)=2$ and $\sigma(4)=1$. The production rate k_i of each gene is modulated by the concentration of the corresponding repressor proteins. In the expression, a_i represents the maximum expression rate, b_i is the Hill coefficient, and H_i is the repressor level that results in half the repression. This model of regulation is appropriate when the repressor binding/unbinding events occur at much faster rates than gene expression.

We model the perturbation caused by the introduction of IPTG in the system at a certain point in time. In our model, IPTG allows the lac promoter to express regardless of the presence of the LacI repressors. This is modelled by the following reaction:

$$\varnothing \xrightarrow{N_1 \cdot I \cdot a_0} P_1$$

where I is a binary variable denoting the presence of IPTG and a_0 is the leak expression rate of the lac promoter when IPTG is present (i.e. when $I = 1$). This rate also accounts for the IPTG concentration, which is not modelled explicitly.

In the above model, we used copy numbers $N_i = 1$ for the SCR and $N_i = 3$ for the LCR[4]. The following parameters were used: $a_i = 1000$ min^{-1}, $b_i = 3$, $H_i = 1$, $d_i^{-1} = 10$ min and $a_0 = 3.5$ min^{-1}. The protein lifetimes were set in accordance with measurements (Taniguchi et al., Science 2010, **329**, 533-538), and a Hill coefficient of $b_i = 3$ was used, since many proteins function in a multimeric form (Xia et al., Proc. Natl. Sci. U.S.A. 2007, **104**, 17329–17334). The parameters a_i and a_0 were tuned for the model dynamics to agree with the empirical data.

We simulated the above model for a duration of 10 h, such that the system state in the measurement window is largely unaffected by the initial protein concentrations (set to $P_i = 0$) (in the measurements, a similar procedure occurs, as cells prior to imaging are cultured for ~10 hours). After this, we simulate the model for further 6 h, sampling the state every 15 min. As in the measurements, the perturbation is performed 3 h after the sampling was started. In each simulation (cell), the two 3 h series prior to and after the perturbation are analysed separately to determine the functionality using the same methods as for the measurement data. A total of 1000 instances of both the SCR and the LCR were simulated to compute the statistics.

Supplementary Figures

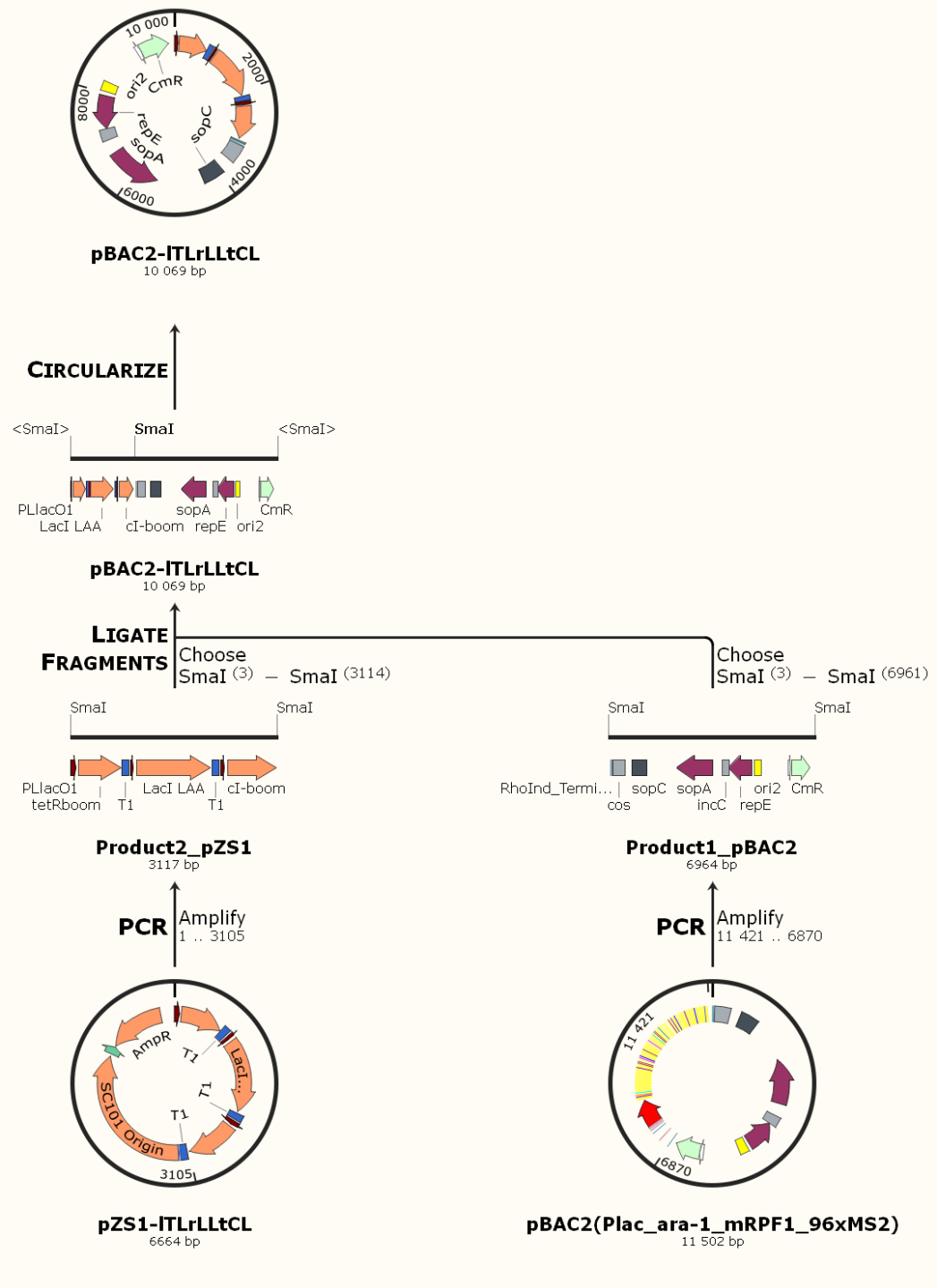

Fig. S1 Illustration of the construction of the SCR plasmid. The pBAC2-lTlrLLtCL plasmid was engineered by inserting the repressilator cassette into pBAC2 ($P_{lac/ara-1}$-mRFP1-MS2-96x) vector (generously provided by Ido Golding, University of Illinois, USA), containing the single-copy origin of replication. The construction history was generated and adapted using SnapGene® 1.5.2.

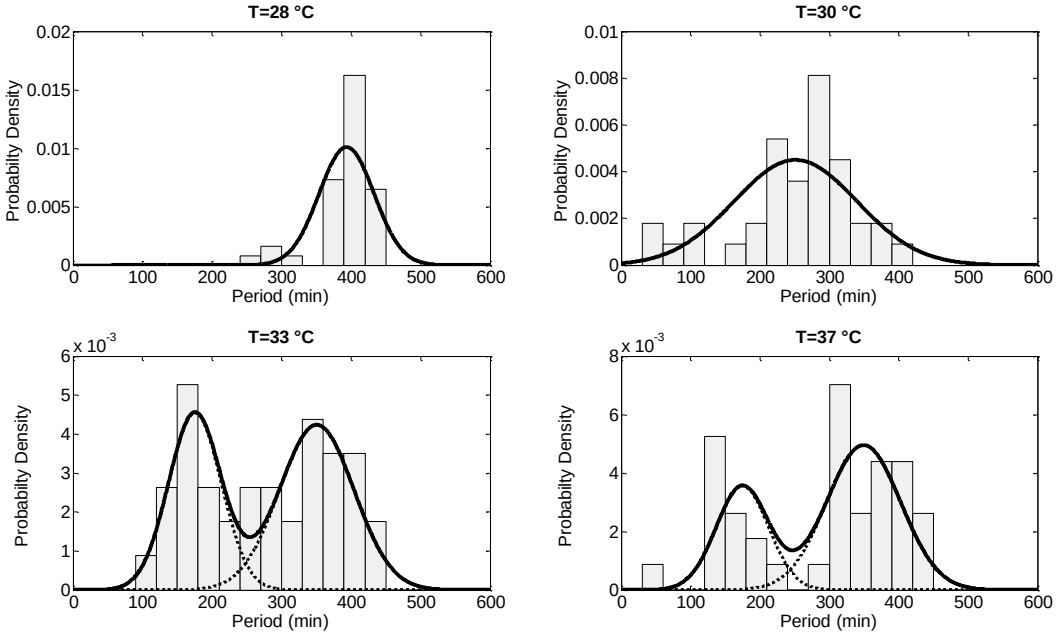

Fig. S2 LCR period distributions at different temperatures. Solid lines represent the probability densities of the fitted model with one or two Gaussians as determined by the likelihood ratio tests. Dashed lines represent the densities of the individual components in the case of two Gaussians. Magnitudes were scaled to represent a probability densities.

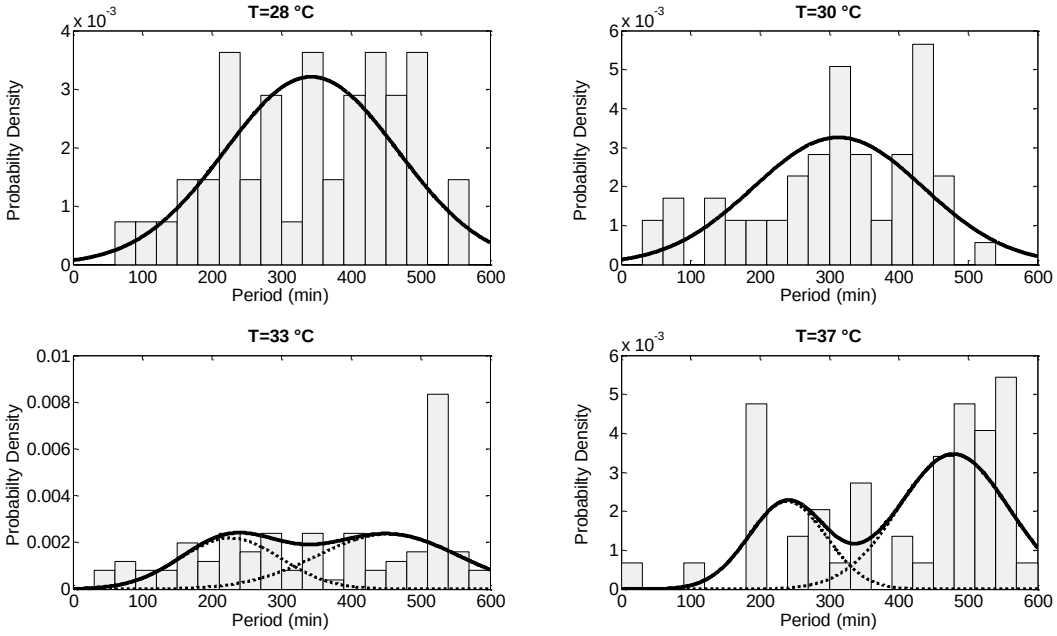

Fig. S3 SCR period distributions at different temperatures. Solid lines represent the probability densities of the fitted model with one or two Gaussians as determined by the likelihood ratio tests. Dashed lines represent the densities of the individual components

in the case of two Gaussians. Magnitudes were scaled to represent a probability densities.

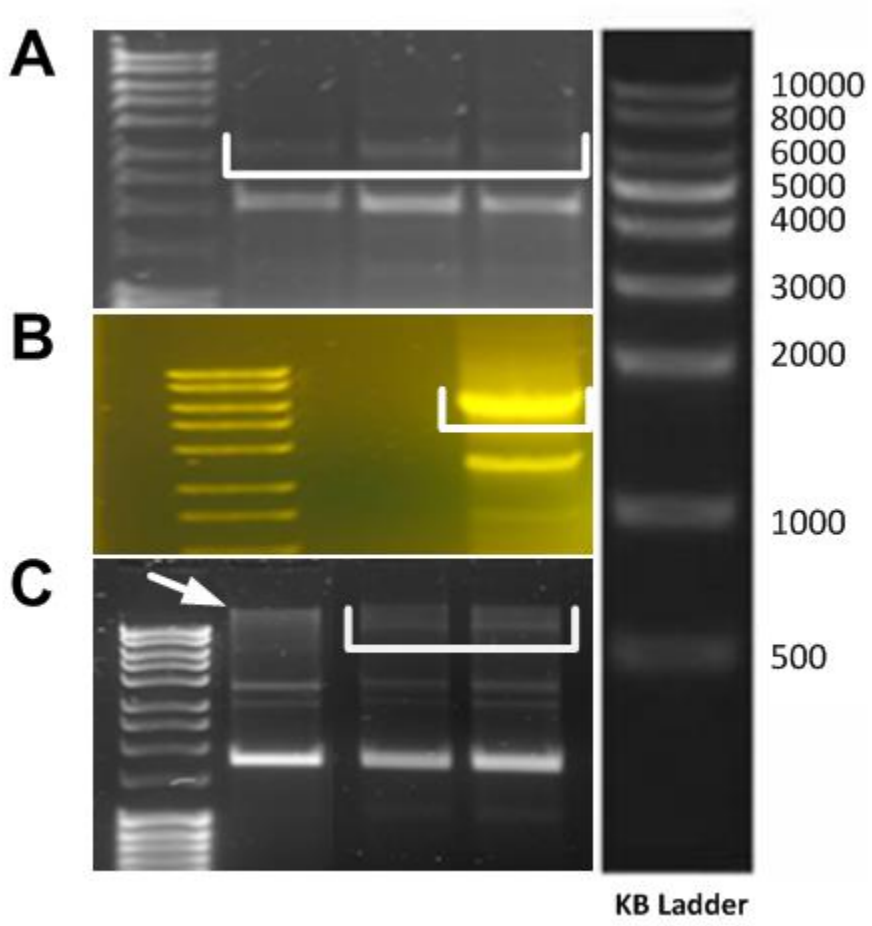

Fig. S4 Split gels for the intermediate steps of the SCR plasmid construction. (A) PCR fragment of 3114 bp amplified from the original pZS1-lTlrLLtCL with appropriate primers, in triplicate (bracket). (B) Lane containing pTB-BAC2 backbone amplified region with the single-copy origin of replication (6961 bp) (bracket). (C) Lane 1: plasmid profile of the strain containing only the reporter plasmid (white arrow). Lanes 2 and 3: two replicates of the plasmid profile of the strain with the reporter and the final construct pBAC2-lTlrLLtCL (SCR plasmid, 10069 bp) (bracket). The numbers of the DNA ladders on the left side of Figures A, B and C are shown on an identical ladder on the right side of the figure.

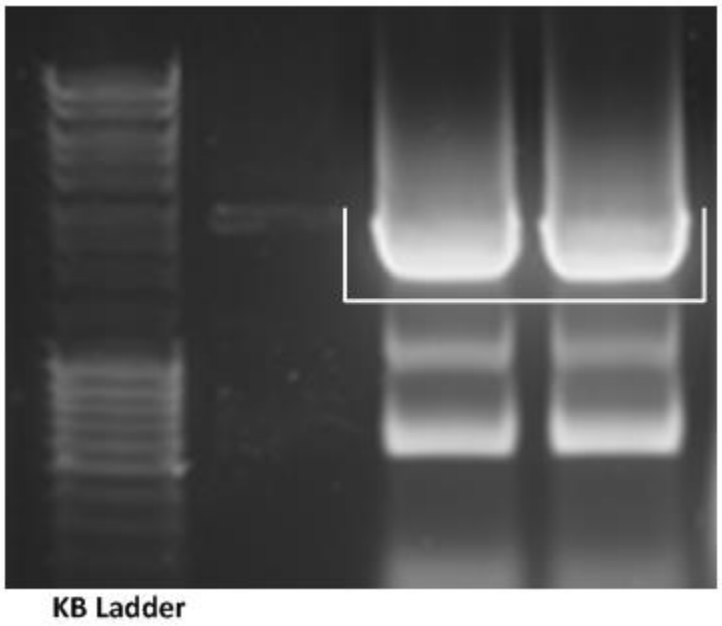

Fig. S5 Split gel of the final product of the SCR plasmid construction. Lane 1: unused. Lanes 2 and 3: PCR amplification of the Repressilator circuit (3114 bp) from the SCR plasmid, pBAC2-lTlrLLtCL, with appropriate primers (bracket). The ladder is identical to those in Fig. S4.

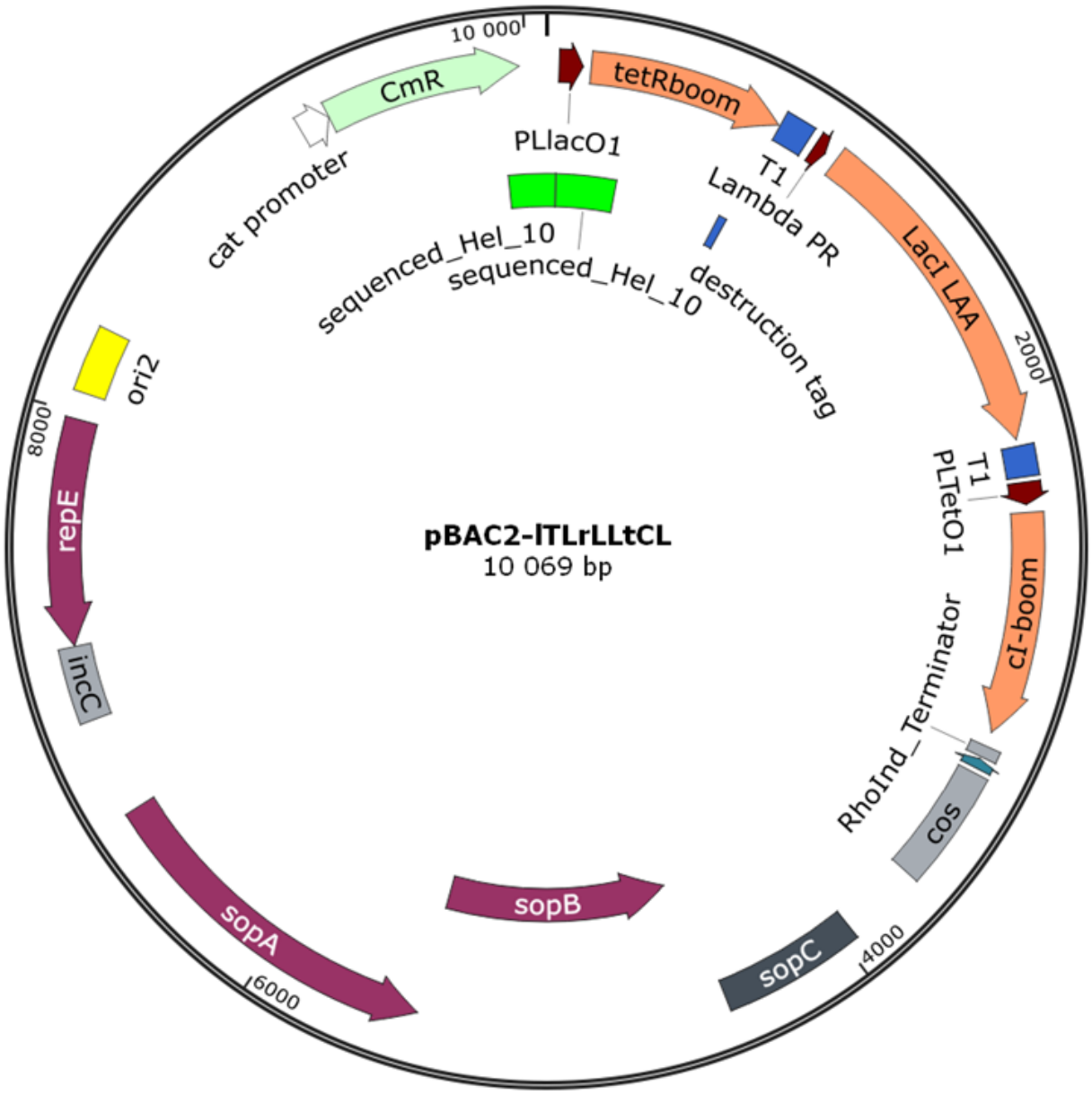

Fig. S6A. Complete map of the single-copy plasmid pBAC2-ITLrLLtCL containing the Repressilator. The green region ('sequenced_Hel_10') corresponds to the sequence in Fig. S6B.

```
Query 1125 GATGGCTTCCATGTCGGCAGAATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAG  1184
           |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  4   GATGGCTT-CATGTCGGCAGAATGCTTAATGAATTACAACAGTACTGCGATGAGTGGCAG  62

Query 1185 GGCGGGGCGTAAttttttAAGGCAGTTATTGGTGCCCTTAAACGCCTGGTTGCTACGCC  1244
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 63   GGCGGGGCGTAATTTTTTTAAGGCAGTTATTGGTGCCCTTAAACGCCTGGTTGCTACGCC  122

Query 1245 TGAATAAGTGATAATAAGCGGATGAATGGCAGAAATTCGATGATAAGCTGTCAAACATGA  1304
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 123  TGAATAAGTGATAATAAGCGGATGAATGGCAGAAATTCGATGATAAGCTGTCAAACATGA  182

Query 1305 GAATTGGTCGACGGCCCTAACCCGGGTCGAGAATTGTGAGCGGATAACAATTGACATTGT  1364
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 183  GAATTGGTCGACGGCCCTAACCCGGGTCGAGAATTGTGAGCGGATAACAATTGACATTGT  242

Query 1365 GAGCGGATAACAAGATACTGAGCACATCAGCAGGACGCACTGACCGAATTCATTAAAGAG  1424
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 243  GAGCGGATAACAAGATACTGAGCACATCAGCAGGACGCACTGACCGAATTCATTAAAGAG  302

Query 1425 GAGAAAGGTACCATGTCCAGATTAGATAAAAGTAAAGTGATTAACAGCGCATTAGAGCTG  1484
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 303  GAGAAAGGTACCATGTCCAGATTAGATAAAAGTAAAGTGATTAACAGCGCATTAGAGCTG  362

Query 1485 CTTAATGAGGTCGGAATCGAAGGTTTAACAACCCGTAAACTCGCCCAGAAGCTAGGTGTA  1544
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 363  CTTAATGAGGTCGGAATCGAAGGTTTAACAACCCGTAAACTCGCCCAGAAGCTAGGTGTA  422

Query 1545 GAGCAGCCTACATTGTATTGGCATGTAAAAAATAAGCGGGCTTTGCT  1591
           |||||||||||||||||||||||| ||||||||||||||||||||
Sbjct 423  GAGCAGCCTACATTGTATTGGCATGTGAAAAATAAGCGGGCTTTGCT  469
```

Fig. S6B. Alignment of sequence obtained from sequencing, using the primer CmR-1-F, against the expected sequence of the original Repressilator[2]. The alignment shows that the single-copy vector is present and the Repressilator has been inserted into it.

GGGTCGAGAATTGTGAGCGGATAACAATTGACATTGTGAGCGGATAACAAGATACT
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CAATTCAGAGTAATAAACTGTGATAATCAACCCTCATCAATGATGACGAACTAACCC
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Fig S6C. The complete sequence of the single-copy plasmid containing the Repressilator, as determined by sequencing.

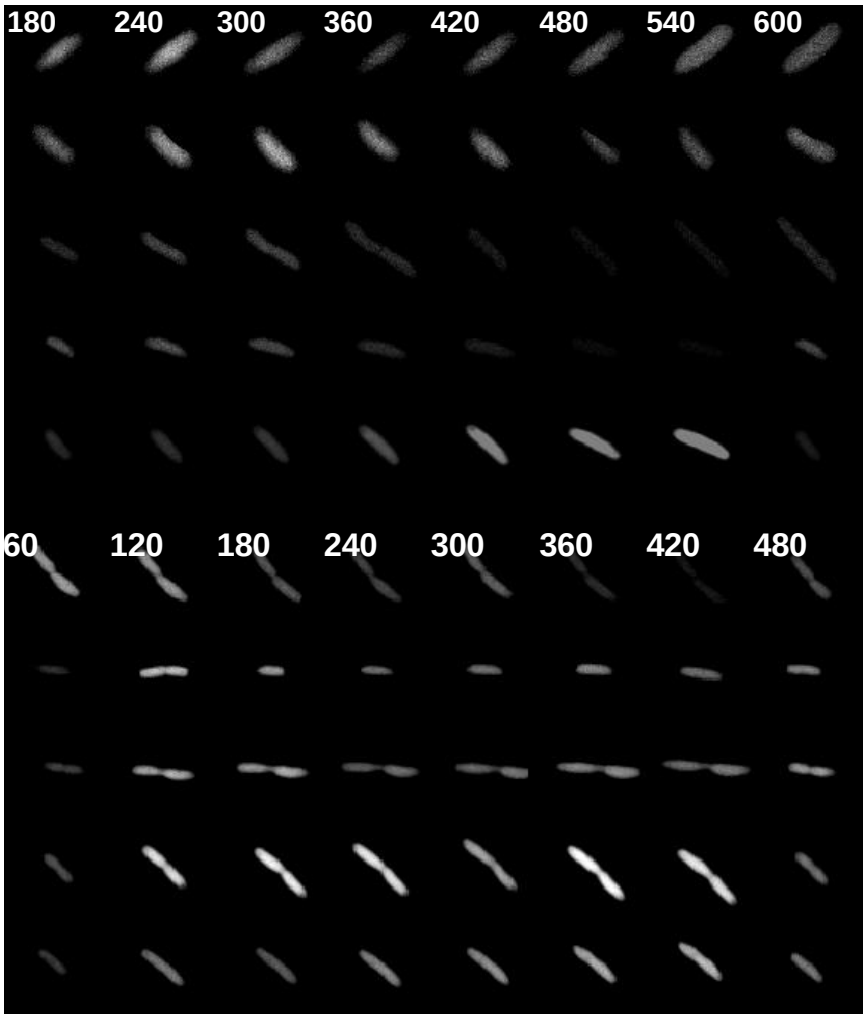

Fig S7. Example images of cells at 30 °C exhibiting oscillatory fluorescence levels. For each cell, 8 frames are shown along with the time after starting the imaging in minutes. From top to bottom, the first 5 cells contain a LCR while the last 5 cells contain a SCR.