

Light-driven synchronization of optogenetic clocks

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Abstract

Synthetic genetic oscillators can serve as internal clocks within engineered cells to program periodic expression. However, cell-to-cell variability introduces a dispersion in the characteristics of these clocks that drives the population to complete desynchronization. Here we introduce the optorepressilator, an optically controllable genetic clock that combines the repressilator, a three-node synthetic network in *E. coli*, with an optogenetic module enabling to reset, delay, or advance its phase using optical inputs. We demonstrate that a population of optorepressilators can be synchronized by transient green light exposure or entrained to oscillate indefinitely by a train of short pulses, through a mechanism reminiscent of natural circadian clocks. Furthermore, we investigate the system's response to detuned external stimuli observing multiple regimes of global synchronization. Integrating experiments and mathematical modeling, we show that the entrainment mechanism is robust and can be understood quantitatively from single cell to population level.

eLife assessment

This study presents a light-entrainable synthetic oscillator in bacteria, the optorepressilator. The authors develop a toolbox using optogenetics that makes the cellular oscillator easily controllable. This toolbox is **valuable**, contributing both to bioengineering and to the understanding of biological dynamical systems. The comparison with a mathematical model, population, and single-cell measurements demonstrate **convincingly** that the planned system was achieved and is suitable to control and study biological oscillators.

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Introduction

Genetic clocks keep time within living organisms in order to program periodic behaviors like circadian oscillations. An intensive genetic analysis of the complex landscape of these biological rhythms has revealed that, from bacteria and fungi to plants and animals, these clocks share similar motifs in the underlying gene regulatory networks [1, 2]. From a reverse perspective, synthetic biologists have tackled the problem of designing from scratch minimal gene networks that can produce periodic patterns of gene expression [3–6]. Very early numerical simulations had shown that networks with an odd number of cyclically connected genes exhibit robust oscillations [7]. The repressilator was the first experimental realization of a synthetic genetic clock based on this principle [3]. As shown schematically in **Fig. 1**, the repressilator is a minimal network consisting of three transcription factors mutually repressing on a loop. The original design, using the repressors LacI, TetR and cI in *E. coli*, displayed marked oscillations in single cells, although with large fluctuations in both amplitude and period. This noise has been recently significantly reduced by genetic optimization, resulting in much more robust albeit slower oscillations [8]. This refactored repressilator is usually referred to as repressilator 2.0 [9]. In this system, the decrease in protein concentration is driven only by growth-related dilution, not by active protein degradation. As discussed in [3], a symmetric, protein only model already predicts limit cycle oscillations provided repression is steep enough:

$$\dot{x} = \frac{\beta}{1 + z^n} - \alpha x \quad \dot{y} = \frac{\beta}{1 + x^n} - \alpha y \quad \dot{z} = \frac{\beta}{1 + y^n} - \alpha z \quad (1)$$

where x , y , z are the three repressors concentrations in units of the dissociation constant K , the concentration of proteins required to repress a promoter to half maximum, α is a common decay rate set by dilution and β is the normalized maximum production rate. In the digital approximation (n), when $\beta/\alpha \gg 1$ the resulting dynamics look like relaxation oscillations with amplitude β/α and a full cycle period given by $T = 3 \log(\beta/\alpha)/\alpha$ (see Supplemental Information and **Fig. 1a**). As a result, although individual cells oscillate indefinitely, the natural dispersion of growth rates α results in a dispersion of periods leading to progressive dephasing of individual oscillators as shown by the simulations in **Fig. 1b**. In the same figure we report an experimental observation of this damping that we obtained using *E. coli* bacteria carrying the repressilator 2.0 [8]. This system contains three fluorescent reporters whose expression is regulated by the same three transcription factors of the repressilator, so that the reporters' concentration follows that of the repressor transcribed by the same promoter (see **Fig. 1a**). The cultures were initially synchronized chemically with IPTG, which deactivates the LacI repressor eliminating the edge between x and y from the network in **Fig. 1a**. This new topology admits a stable fixed point where the concentrations of x and y are maximal while z goes to zero. Next, the cultures were maintained in exponential growth phase by periodic dilutions in a multiwell plate while the population-averaged fluorescence signal was monitored using a plate reader (see Materials and Methods). For clarity reasons, we will only report the fluorescence signal from the CFP reporter that indirectly quantifies the concentration of the TetR repressor or y in our simple model. **Fig. 1b** shows that when starting from the synchronized state, the population signal from the CFP reporter displays high contrast oscillations with a period of 16 hours and an amplitude that is reduced to a half after about 2.5 periods. Phase drifts are very common in natural biological oscillators. Small couplings within a population of oscillators can give rise to a globally synchronized state [10], as in the case of cardiac pacemaker cells [11] or quorum sensing coupled genetic oscillators [12, 13]. Other genetic oscillators, like circadian clocks, rely instead on a periodic external cue (zeitgeber) to constantly adjust their phase to that of a common environmental cycle. Sunlight is the predominant zeitgeber of natural circadian clocks [14]. In the context of synthetic biology, light is a particularly versatile input for controlling the state of genetic circuits and programming gene expression in space and time with much greater precision

than chemical signals [15–18]. Optical signals can also be multiplexed through spectral [19, 20] or amplitude modulation [21]. A growing number of optogenetics tools have been recently applied in prokaryotes to control different aspects of bacterial physiology [22], such as growth [23–25], antibiotic resistance [26], motility [27, 28] and adhesion [29]. A widely used optogenetic system is the light-switchable two-component system, CcaS-CcaR from *Synechocystis* PCC 6803 [30]. CcaS senses green light and phosphorylates the actuator CcaR. Phosphorylated CcaR binds to the promoter $P_{cpcG2-172}$, activating gene expression. Red light reverts CcaS to the inactive state and shuts down transcription from $P_{cpcG2-172}$. This system was optimized for controllable gene expression with a high dynamic range in *E. coli* [31, 32].

Here we show that integrating an optogenetic module in the repressilator circuit enables the use of light to synchronize, entrain, and detune oscillations in gene expression within single cells or entire populations. We employ the CcaS-CcaR light-inducible system to express one of the repressilator proteins, resulting in a novel 4-node optogenetic network named the ‘optorepressilator’. With this modification, light acts as a zeitgeber, enabling precise phase adjustments among synthetic genetic clocks within individual cells, leading to persistent population-wide oscillations. We show that a population of these synthetic oscillators can be synchronized through transient green light exposure or be entrained via a sequence of short pulses, sustaining indefinite oscillations. Additionally, we explore the system’s response to detuned external stimuli, revealing multiple synchronization regimes.

Results

Optorepressilator: a light-controllable repressilator

In the optorepressilator (Fig. 2a), LacI proteins are produced by two genes. We indicate with x the normalized concentration of the repressilator’s LacI, transcribed via the λ promoter and repressed by z (cI), and with x' the concentration of LacI proteins transcribed via the light-inducible promoter $P_{cpcG2-172}$ [31] (Fig. 2b). x and x' add up to repress protein y , while a light-dependent production rate drives x' dynamics:

$$\dot{x} = \frac{\beta}{1 + z^n} - \alpha x, \quad \dot{y} = \frac{\beta}{1 + (x + x')^n} - \alpha y, \quad \dot{z} = \frac{\beta}{1 + y^n} - \alpha z, \quad \dot{x}' = \beta' - \alpha x' \quad (2)$$

where β' is a light dependent production rate. We will treat β' as a two-valued function with β_R the low value under red light and β_G the high value under saturating green light. It is important to note that the dynamics of x' is decoupled from all other repressors and only determined by light. In particular, under steady illumination conditions $x' = \beta'/\alpha$. If the expression of the light-inducible LacI is adjusted to have

a dynamic range containing the dissociation constant K , then we might be able to optically switch the system from limit cycle oscillations under red light to a fixed point under green light (Fig. 2c). In the digital approximation, the fixed point is attained when $\beta_G/\alpha > 1$ and it has coordinates $x_0 = 0$, $y_0 = 0$, $z_0 = \beta/\alpha$.

Fine-tuning optogenetic expression

Estimates for the LacI dissociation constant K vary between 0.01–0.1 nM [33]. Even taking into account the fact that the repressor plasmid is present in more than one copy (5 copies), this suggests that a small number of LacI proteins may already disrupt the limit cycle. Therefore, controlling the leakage of the promoter under the red light is crucial. The optogenetic module consists of two components, named as sensor and actuator in Fig. 3c. In order to finetune the expression of LacI, we created four different versions of the actuator in which both transcription and translation were modulated on two levels. Translation was controlled by substituting the

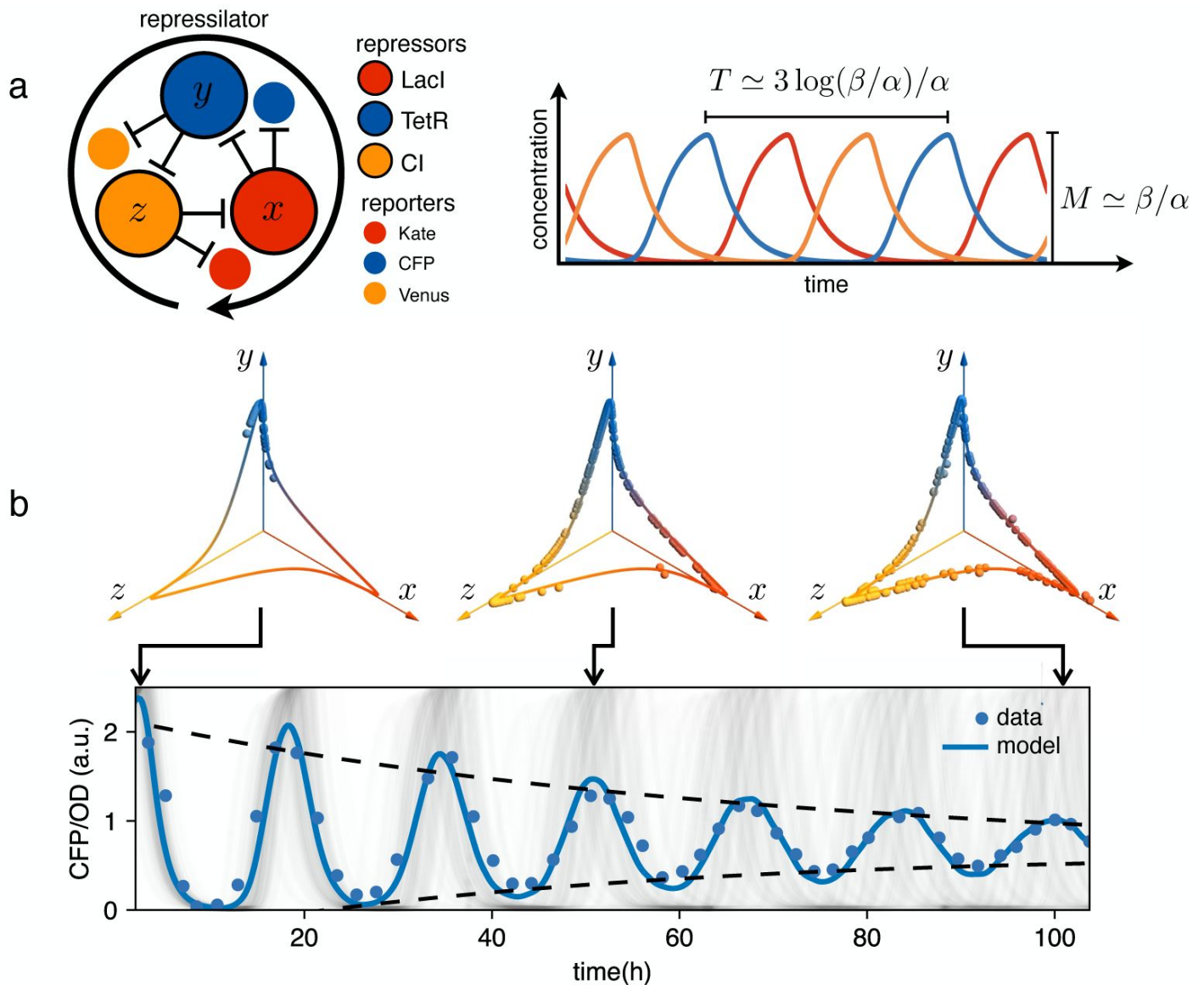


Fig. 1

Dephasing in a population of repressilators.

a (left) Schematic representation of the repressilator 2.0 plasmid. (right) Simulated time evolution of the three repressors concentrations in the limit cycle of the protein-only model in Eq. 1. The reported expressions for amplitude and period are derived in the limits $n \rightarrow \infty$ and $\beta/\alpha \gg 1$ (see Supplementary Material). **b** Concentration of the reporter CFP (TetR) as a function of time for an initially synchronized population of oscillators growing in a multi-well plate. Blue circles represent average over two technical replicates, corresponding errorbars are comparable to symbol size. The shadowed gray curves show simulations of independent oscillators with parameters $n = 4$, $\beta = 87 \text{ h}^{-1}$ and α extracted from a log-normal distribution of mean 0.73 h^{-1} and standard deviation 0.029 h^{-1} . Dephasing due to the variability of oscillators periods causes a damping in the mean population signal reported as a blue line. Snapshots of the population ensemble in the 3D concentration space x - y - z are reported for three instants of time during the simulation, clearly showing progressive dephasing of individual oscillators over the limit cycle trajectory.

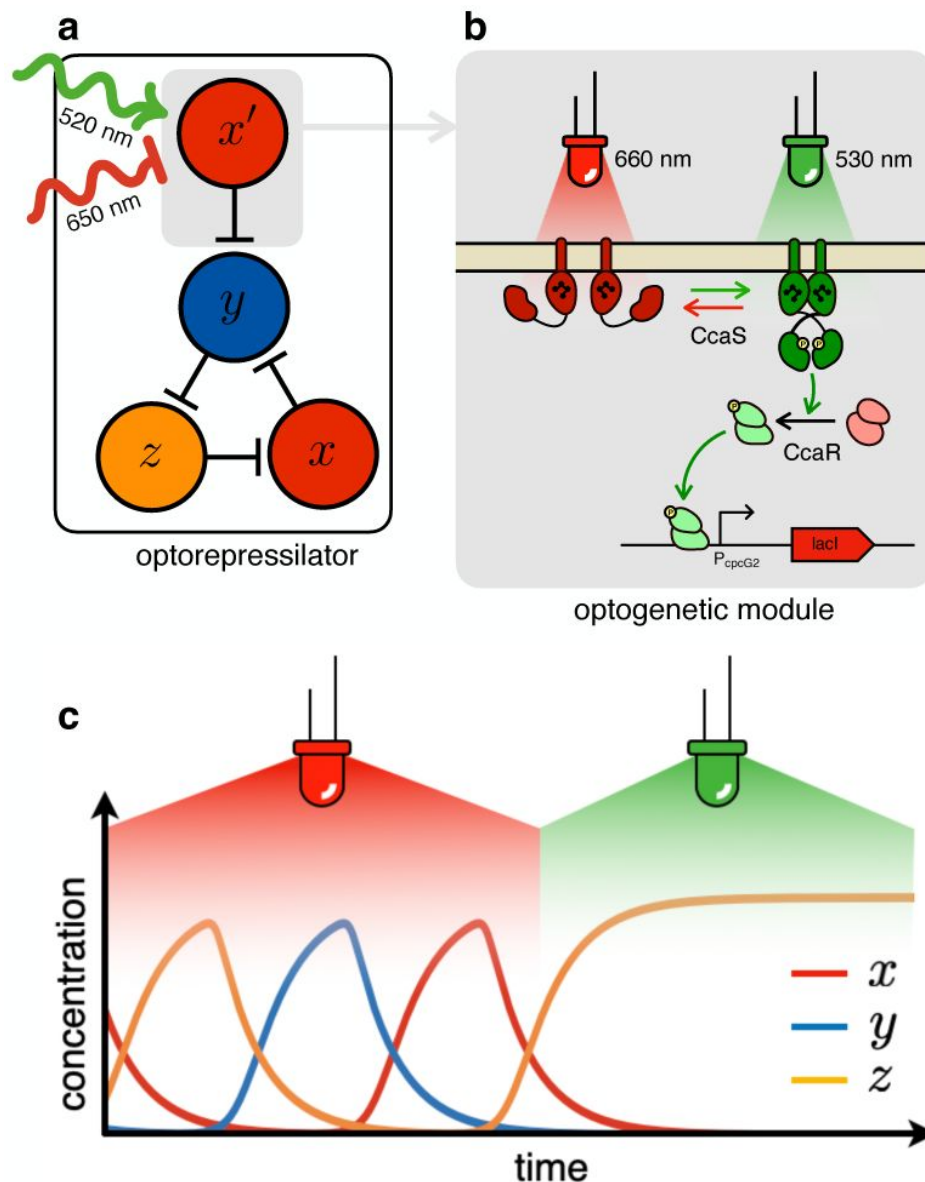


Fig. 2

The optorepressilator: a light-controllable genetic oscillator

a) Schematic illustration of the optorepressilator circuit. Green and red light respectively promote and repress the production of one of the three transcription factors in the repressilator (*LacI*). **b)** Working mechanism of the optogenetic module. A light-driven two-component system controls the production of an additional copy of *lacI*. The optogenetic system consists of the membrane-associated histidine kinase CcaS and its response regulator CcaR. Absorption of green light increases the rate of CcaS autophosphorylation and phosphate transfer to the transcription factor CcaR. Phosphorylated CcaR promotes transcription of an additional copy of the *LacI* gene from the promoter $P_{cpcG2-172}$. Red light reverts CcaS to the inactive state and shuts down transcription from $P_{cpcG2-172}$. **c)** Ideal optorepressilator's dynamics according to Eq. 2. The system oscillates unperturbed under red light, but collapses to a fixed point under green light when the extra x' represses y .

ribosome binding site (RBS) (BBa B0034) in the original plasmid with a weaker RBS (BBa B0033) from the same IGEM Community collection. Transcription was modulated through gene copy number by moving the light-driven gene expression cassettes from the plasmids to the genome. Combined with the sensor and actuator plasmids, these constructs resulted in four versions of the optogenetic module (**Fig. 3a**) with different expression ranges. We characterized these expression ranges as a function of incident light (**Fig. 3b**) using sfGFP as fluorescent reporter and a custom-made light-addressable multiwell plate (see Supplemental information). By comparing expression at maximum green level, we can estimate that RBS substitution leads to a 27-fold decrease in reporter production (**Fig. 3b**), while relocation of the light-driven cassette to the genome reduces expression by 57-fold. The combination of weak RBS and genome insertion results in a gene expression range below our instrument sensitivity (estimated combined fold reduction of 1500). We then replaced the sfGFP gene with LacI in all four versions of the optogenetic module and transformed the repressilator plasmid (pLPT234) in each of the four strains. As a first step, we verified that the oscillations of the limit cycle were preserved under red light. To this end, we first chemically synchronized the oscillations with IPTG and then monitored the concentration of TetR (y) reported by CFP. Cultures were maintained in exponential phase by periodic dilutions in multiwells under constant red light. We found that only the strain with the lowest expression level (represented in **Fig. 3c**) oscillated with the same amplitude and period as the control strain containing only the repressilator plasmids (**Fig. 3d**). In contrast, no clear oscillations were observed in the other three strains, where LacI leaking from the red-repressed promoter destroys the limit cycle and collapses all cells in a fixed point where the repressilator protein cI is high and TetR is low.

Optogenetic synchronization

Having verified that the system oscillates under red light, we then checked whether green light can synchronize a population of optorepressilators. **Fig. 4a** shows the population signal of CFP fluorescence reporting the concentration of TetR (y) in multiwell cultures. The cultures were constantly kept under red light for the first 40 hours. Although individual cells oscillated, their phases were randomly distributed so that the average population signal was constant. At $t = 40$ h we switched from red to green for 12 hours, and CFP fluorescence decayed to zero. This is expected when extra LacI is produced by the optogenetic module, repressing both TetR and CFP. When the population signal decayed completely, all the cells were stuck in the same fixed point ($x_0 = 0$, $y_0 = 0$, $z_0 = \beta/\alpha$ in the model), so that when switching back to red, they started oscillating in synchrony. This was also confirmed by single-cell data from a mother-machine microfluidic chip (**Fig. 4b**). Under the same light protocol as in **Fig. 4a**, most of the channels blinked in unison after exposure and removal of synchronizing green light (Supplemental Video 1).

Optogenetic entrainment

Both plate reader and mother-machine experiments showed that cells carrying the optorepressilator system could be synchronized by transient light exposure. Oscillations in the population signal, however, were progressively dampened again by the dispersion of growth rates and thus of individual clocks' periods. This was particularly evident in the mother-machine experiment, where the growth rate variability was larger. Natural genetic clocks are able to counteract period dispersion by exploiting an external periodic stimulus to advance or delay their phases. For example, a one-hour exposure to light at bedtime can delay the human circadian rhythm by about two hours, while exposure to light after waking up can advance our schedule by a little less than half an hour [34]. Interestingly, the optorepressilator model in equation (2), despite its simplicity displays a very similar behavior. In **Fig. 5a** we report the phase shift produced by a single pulse as a function of the pulse arrival time t_0 . This type of curve is often referred to as a phase response curve and was obtained here by numerical integration of (2) for a single optorepressilator. Both the pulse arrival time and the phase shift are measured in units of

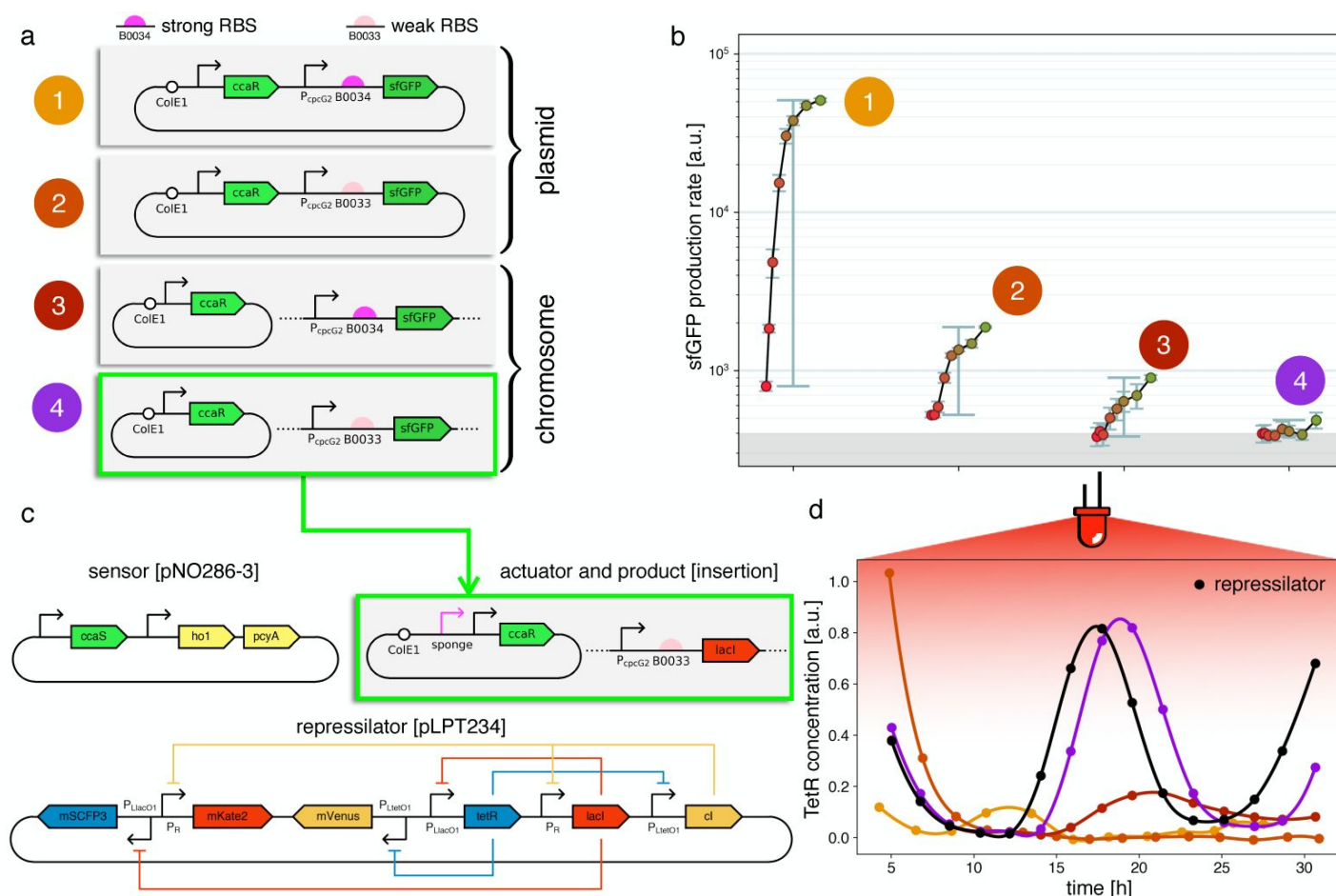


Fig. 3

Fine-tuning gene expression in the optorepressilator circuit.

a To tune expression from the optogenetic module we varied the strength of RBSs and gene copy number by placing the output gene (sfGFP) either on a plasmid or in the chromosome. **b** Light-controlled expression levels of the sfGFP reporter from the four constructs in **a**. For each curve, the samples were exposed to a fixed level of red light while increasing green light intensity, as indicated by the dots' colors. The main error bars represent the dynamic range of each sample. The smaller error bars represent the standard deviation of individual light conditions between replicates made in three separate days. The gray shaded area represents values of the expression level that we expect to be below our instrument sensitivity. For light intensities, see Supplemental information. **c** Scheme of the final optorepressilator circuit (see Materials and Methods). **d** Time evolution of TetR reporter (CFP) concentration in a population of IPTG synchronized cells growing under red light. Black points from the original repressilator 2.0 display marked oscillations. Colored lines correspond to the four constructs in **a** with sfGFP replaced by LacI and the addition of the sponge promoters as in pSpongeROG. Circles represent data, where each dot is the average of two replicates with error bars comparable to marker size, while lines are spline interpolations. Only the purple line, corresponding to the system in **c**, shows oscillations comparable to those of the original repressilator.

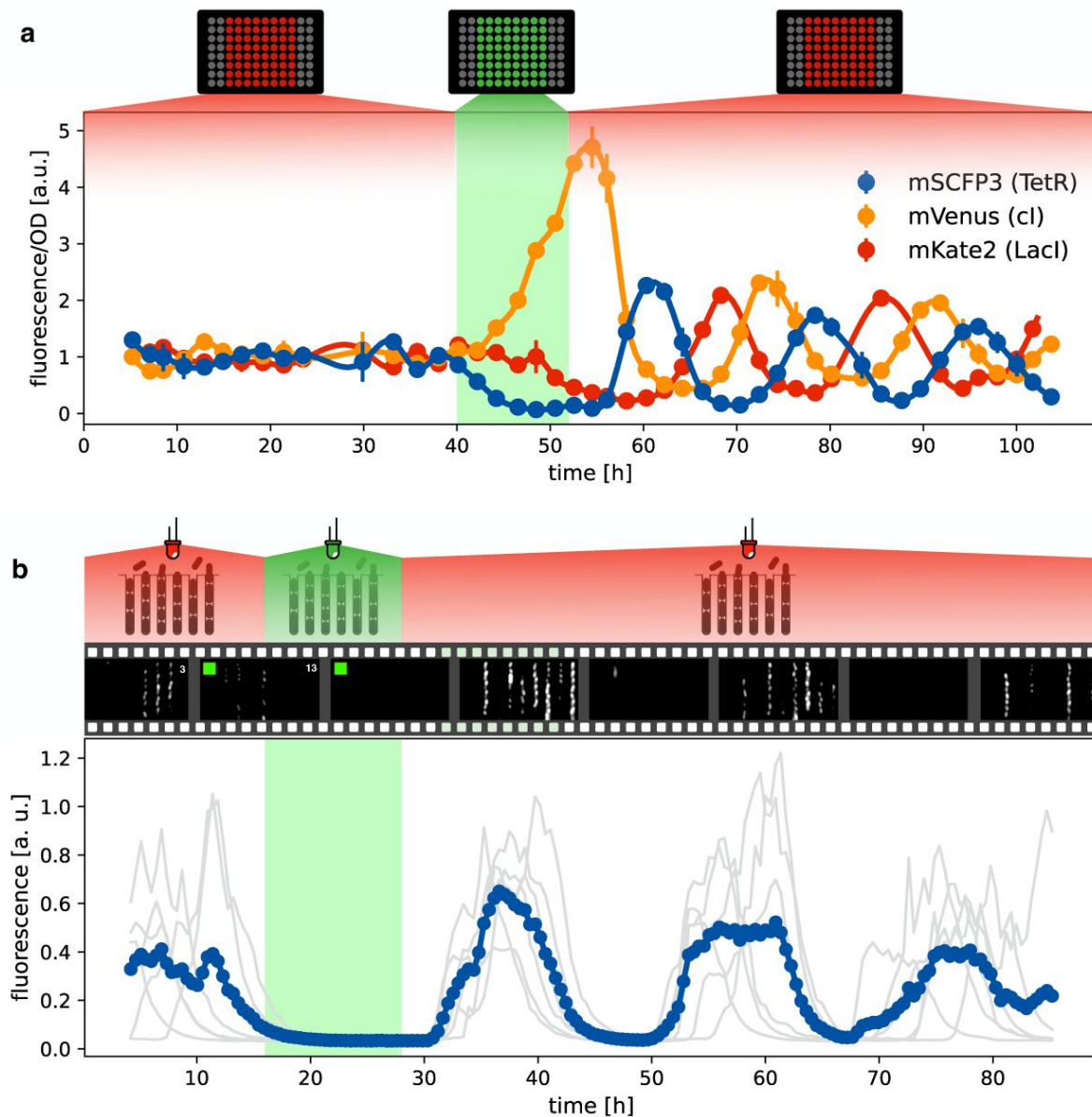


Fig. 4

Optical synchronization.

a Time evolution of mSCFP3(CFP), mVenus and mKate2 signals from a population of exponentially growing cells in multiwell plate. Red and green shaded areas represent the illumination protocol. Dots reports data for optorepressilator cells and clearly shows the appearance of synchronous oscillations after transient illumination with green light, each marker is the average of two replicates and the error bar is data range. **b** Single cell data from a mother machine experiment employing the same light protocol as in **a**. The gray curves represent the concentration of CFP for individual cells growing in different channels, while the blue curve is their average. Snapshots above the plot report fluorescence imaging of bacteria in the microfluidic chip, centered at the corresponding time point on the time axis below.

the free running optorepressilator period T_0 . A positive pulse arrival time $t_0 > 0$ results in a positive phase shift $\Delta > 0$ that delays subsequent oscillations, while a negative t_0 results in a phase advance ($\Delta < 0$).

This behavior can be understood at least qualitatively by simple considerations. When a pulse arrives before y reaches the maximum ($t_0 < 0$), a burst of x' is produced (**Fig. 5b**) that may trigger an earlier decay of y if $\beta_G/\alpha > 1$ and the pulse duration τ is long enough ($\beta_G\tau \gtrsim 1$). This results in a negative phase shift for the subsequent peaks, which is greater the earlier the light pulse arrives. Conversely when the pulse arrives while y is decaying the x' burst will prolong the decay of y until the moment when x' falls below 1. This results in a positive phase shift that increases as t_0 increases. Let us now consider an ensemble of optorepressilators with distributed natural frequencies $\nu_0 = 1/T_0$, subjected to a train of light pulses with repetition frequency f . Each repressilator could be entrained by the external signal if their period could be stretched or compressed such that $(1 + \Delta)T_0 = 1/f$. In other words, entrainment requires that each oscillator in the population receives the external pulses at its own time t_0 such that $\Delta(t_0) = 1/f T_0 - 1$. A monotonically increasing phase response curve also guarantees that this entrained steady state is stable against fluctuations. For instance, suppose a fast oscillator is entrained by a slower train of light pulses ($t_0 > 0$). If a fluctuation in growth rate leads to an increase of the natural oscillation period T_0 , then the next pulse would arrive with a negative t_0 resulting in a negative delay Δ , restoring entrainment. From this simplified theoretical discussion, we deduce that if real optorepressilators had the predicted phase response curve, then it should be possible to use a train of light pulses as a zeitgeber capable of producing long-term oscillations in gene expression at the population level. We first demonstrate this by monitoring the population signal from CFP (reporting TetR or y in the model) in multiwell cultures under constant red illumination interrupted by 2 h pulses of green light with period $T = 18$ h. **Fig. 5c** shows high contrast and undamped oscillations to be contrasted to the damped ones in **Fig. 1b** and **Fig. 4a**. The bottom panel in **Fig. 5c** shows the result of a numerical simulation with the same parameters as in **Fig. 1b** and the addition of a periodic light stimulation. Simulation panel also displays in gray the signals from individual optorepressilators before performing the average (in blue). These results are also confirmed by mother machine observations reported in **Fig. 5d** (Supplemental Video 2). For the simulations in the lower panel of **Fig. 5d**, all parameters remained the same as in **Fig. 5c** with the exception of the standard deviation of the natural periods which was increased from 0.04 to 0.95 to better reproduce the experimental observations in the mother machine. A narrower distribution of growth rates in multiwell cultures could be due to the fact that faster-growing bacteria outperform slower-growing cells in a competing environment, unlike in the mother machine where there is no competition between cells in different channels. The theoretical discussion above predicts that the individual oscillators will each find their own stable phase with respect to external pulses. The slower ones will arrange themselves to receive the pulse on the rising edge of the peak to reduce their period, while the faster ones will have the pulse arriving after the peak to increase their period. We confirm this in both experiments and simulations in **Fig. 5d**, where the individual traces of a slow (higher amplitude) and a fast (lower amplitude) oscillator have been highlighted with dotted and dashed lines respectively.

Detuning

In an entrained population, most oscillators are detuned from their natural frequency to that of the external signal. In this section we will explore detuning by starting with numerical simulations of our simple model in **Eq. 2** and then comparing them with long-term observations of detuned optorepressilators in a plate reader experiment. **Fig. 6a** shows a 3D surface representing the ratio of the actual optorepressilator frequency ν over external frequency f as a function of frequency and amplitude of the external signal β' . The plateaus in this graph are the so-called Arnold's tongues [10], i.e. regions of synchronization in the frequency/amplitude plane. On the cyan plateau, the system oscillates with the same frequency as the external signal, while there are also higher-order plateaus with a fractional value of the ratio f/ν_0 . On the cyan tongue, when the system is detuned to higher frequencies, its phase will be such that it receives the light pulse on

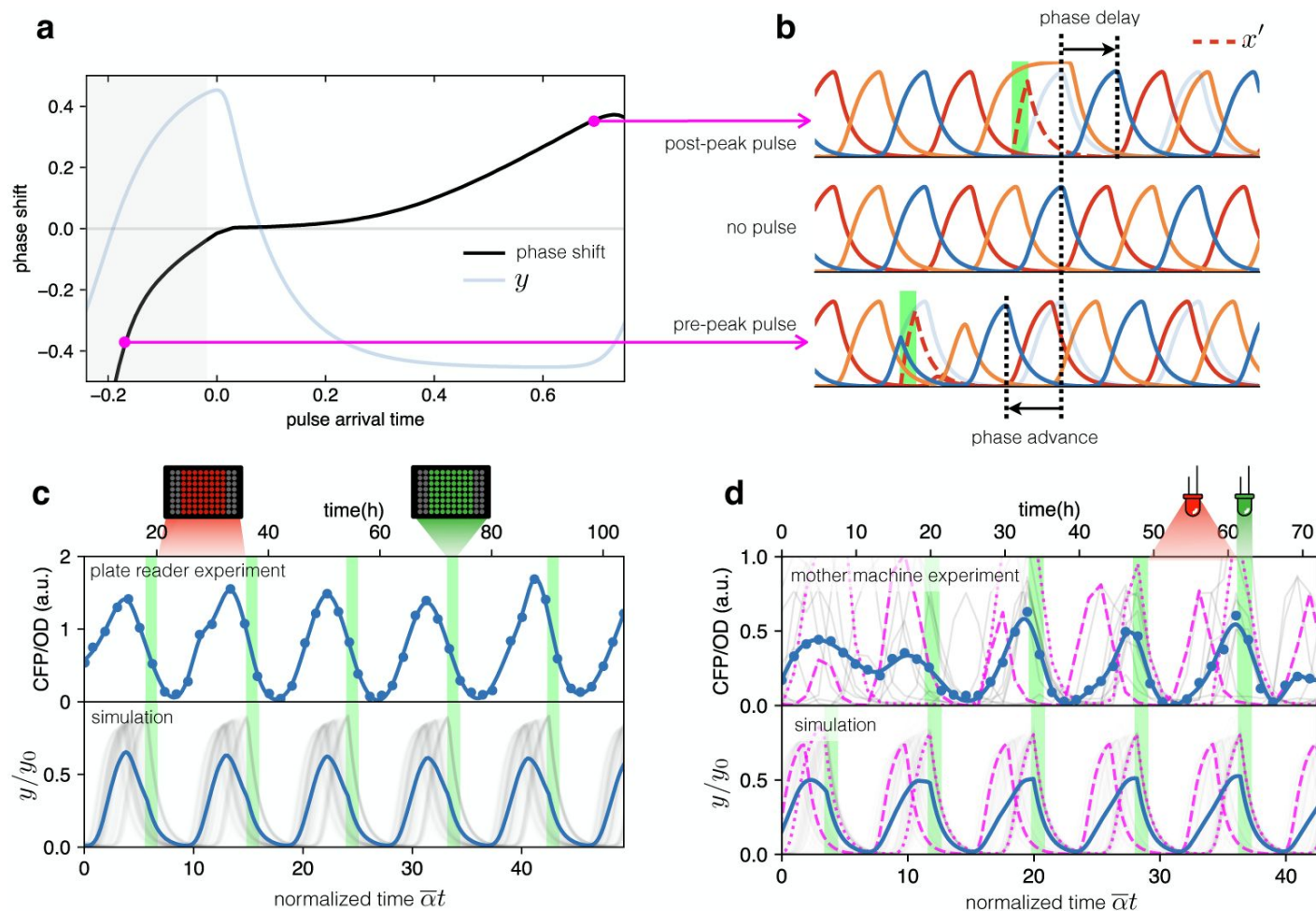


Fig. 5

Optical entrainment.

a Phase shift as a function of pulse arrival time. Both quantities are expressed in units of the free-running optorepressor period. **b** Numerical simulations of equation (2) illustrating the effect of pulse arrival on the phase of optorepressor oscillations. **c** (top) CFP signal from a population of cells growing exponentially in multiwells that are exposed to a train of periodic pulses of green light. The population signal displays undamped oscillations demonstrating optical entrainment. (bottom) Numerical simulation of an ensemble of optorepressors evolving according to equation (2) and with distributed growth rates (same parameters as in Fig. 1b). Gray lines are individual oscillators while blue is the population average. **d** (top) Gray lines are CFP signals from single cells growing in a mother machine under periodic pulsed illumination. The blue curve is the average. (bottom) Numerical simulation as in c but a larger dispersion of growth rates is required to match experiments above. In both data and simulation the magenta dotted curve highlights a slow oscillator (low growth rate and larger amplitude) whose phase is adjusted to receive the peak on the rising edge to anticipate the next oscillation. Magenta dashed lines highlight faster oscillators whose phase is such to receive the pulse on the decaying edge to delay the following oscillation.

the rising edge of the y protein. When detuned to lower frequencies, however, it shifts to receive pulses on the falling edge of y . The width of this tongue is about 0.8 to 1.5 of ν_0 . When trying to detune to even lower frequencies, the system goes to an higher order synchronization tongue where it oscillates at twice the frequency of the external signal. Similarly, forcing to higher frequencies moves the system to fractional order tongues, such as the green one in the plot, in which the system oscillates at half the external frequency. All these regimes are found in experiments in which we maintain cultures of optorepressilators in exponential phase within multiwell plates that are independently addressed with light signals of different frequencies (Fig. 6b). It is also remarkable to observe how the relative phase between the system and the external signal evolves within the cyan tongue, as predicted by the model. A discrepancy is apparently found in the green tongue, where we experimentally observe oscillations at the same frequency as the external signal. This is not unexpected, however, when one realizes that although the individual optorepressilators may oscillate at half the external frequency f , they can peak at either the even or odd peaks of the external signal. When we average these alternating peaks over the population we get a small amplitude oscillation at the frequency of the external signal. This is also confirmed by a mother machine experiment where we tried to detune to about twice the natural frequency and observed different channels oscillating at half the signal frequency and with alternating peaks (Fig. 6c).

Discussion

Inspired by the light entrainment of natural circadian rhythms, we designed the optorepressilator, a four-node synthetic oscillatory network that can lock its phase to that of a periodic light signal. By integrating an optogenetic module into a repressilator circuit, we demonstrate, theoretically and with experiments, that light can synchronize, entrain, and detune oscillations within single cells or an entire population. We find that this four-node network shows a phase-sensitive response to light pulses. Depending on the arrival time, a pulse can either delay or advance the clock as in many natural circadian clocks. As a result, our system can be entrained by periodic light pulses, even when the forcing period deviates from the natural frequency. Using a combination of optogenetic experiments ranging from the macroscopic population scale to the microscopic scale of single cells, we show that the entrainment mechanism is robust and can be understood quantitatively by a simple protein-only model.

The optorepressilator enriches the toolbox of genetic modules by adding a light-synchronizable clock that could be used to program precise oscillatory patterns of gene expression. When compared to chemical signaling, the use of light to control and coordinate the functioning of synthetic networks offers clear advantages in terms of spatial and temporal modulation. Light cues can be applied and removed on the spot, without waiting for media substitution [35]. In addition, using spatial light modulators it could be possible to address spatially separated subpopulations within a common environment to oscillate with distinct periods and phases. Optical synchronization of genetic oscillators can be easily implemented in a wide range of experimental settings like liquid cultures, agar plates or microfluidics. Light, for example, can penetrate bioreactors and orchestrate gene expression in a population of uncoupled oscillators. Moving to red inducible optogenetic systems, optical signals could also penetrate biological tissues for *in vivo* applications [9]. Finally, at the fundamental level, the optorepressilator enables the quantitative investigation of response in genetic clocks to find, in a synthetic biology context, a universal phenomenology in the physics of nonlinear phenomena, such as the presence of multiple synchronization states separated by Arnold tongues [36].

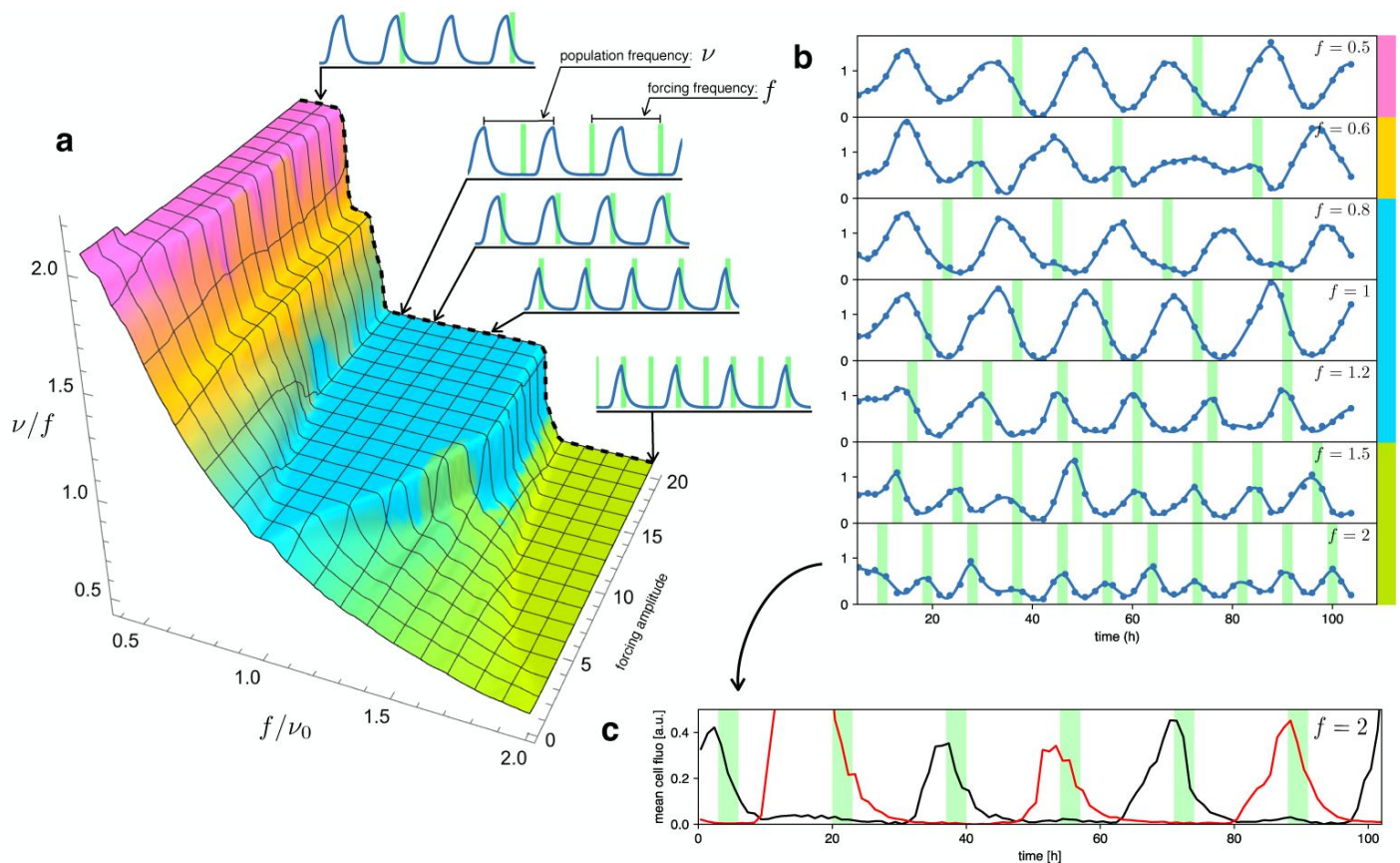


Fig. 6

Detuning.

a 3D surface representing the ratio between the actual frequency of a single forced optorepressilator and the frequency of the forcing signal, as a function of the forcing frequency and amplitude. The colors of the plateaus (Arnold's tongues) highlight different regions of global synchronization in the frequency/amplitude plane. **b** Time evolution of the average CFP concentration (blue curve) in a plate reader experiment, for different frequencies of green light pulses (green bars). The color bars at the right of the plot indicates the corresponding region on the surface in **a**. **c** Mother machine experiment revealing two distinct cells oscillating at half the signal frequency but peaking on alternate pulses of incoming light. This explains why for $f = 1.5$ and $f = 2$ in **b** we observe a population mean oscillating with the same frequency of the forcing instead of the $\nu/f = 0.5$ value predicted for the green tongue in **a**.

Materials and Methods

Strains and plasmids

A list of the strains and plasmids used is in Supplemental Table 1 and 2, along with plasmids maps and their description. Genome insertion of light-driven cassettes was performed using lambda-red recombination [37] targeting the *attB* genome site. The starting strain was *E. coli* DHL708 (a gift from Johan Paulsson, Addgene plasmid # 98417) [8] for all insertions. All the recombination cassettes were amplified from modified versions of plasmid pKD46 [37] (Supplemental Table 2). In addition to kanamycin antibiotic resistance, the recombination cassettes contained the desired protein gene (LacI, sfGFP) controlled by the light-driven promoter $P_{cpcG2-172}$. Plasmid cloning was performed using In-Fusion cloning (Takara Bio) for large rearrangements and Q5® Site-Directed Mutagenesis Kit (New England BioLabs) for small modifications *i. e.* RBS changes. The final optorepressilator system is composed of: the strain MCC0233 obtained inserting $P_{cpcG2-172}$ (the light-driven promoter), a weak RBS and LacI open reading frame in the genome of DHL708; pNO286-3 (a gift from Jeffrey Tabor, Addgene plasmid # 107746; <http://n2t.net/addgene:107746>; RRID:Addgene 107746) [32], plasmid with mini-CcaS light sensor and the enzymes for the phycocyanobilin chromophore; pLPT234 (a gift from Johan Paulsson, Addgene plasmid # 127855; <http://n2t.net/addgene:127855>; RRID:Addgene 127855) [9], plasmid with the core repressilator circuit and reporters; pSpongeROG, a modified version of plasmid pSR58.6 (a gift from Jeffrey Tabor, Addgene plasmid # 63176; <http://n2t.net/addgene:63176>; RRID:Addgene 63176) [19] added with the sponge from plasmid pLPT145 (a gift from Johan Paulsson, Addgene plasmid # 85527; <http://n2t.net/addgene:85527>; RRID:Addgene 85527) [8] and without the light-driven gene expression cassette (sfGFP);

Plate reader experiments to follow circuit oscillations

Bacterial strains were grown from glycerol stock for 16 hours in LB with appropriate antibiotics (see Supplementary Table 3 for antibiotic working concentration), in a falcon tube exposed to saturating 660 nm red light in a shaker incubator (200 rpm) at 37 °C. Cultures were diluted $1 : 10^6$ approximately 8 hours before the beginning of the experiment in imaging medium [8] with antibiotics under red light, and 1 mM IPTG was added in this interval if samples had to be chemically synchronized. Samples were transferred in duplicates to a 96-well plate (Greiner GR655096), and we measured OD600 and fluorescence for sCFP, mVenus and mKate2. When OD600 reached approximately 0.8 for the first time, the samples were diluted 1:4 in new wells with imaging medium plus antibiotics. Every two hours, we measured OD600 and fluorescence and diluted about 1:4 in fresh culture medium plus antibiotics to bring the cultures back to an OD of 0.2 and keep the bacteria under exponential growth conditions. OD600 and fluorescence were measured with a TECAN Infinite M Nano+ plate reader warmed to 37 °C. The parameters to detect fluorescence were: mSCFP3 excitation 433 (9) nm emission 474 (20) nm; mVenus excitation 500 (9) nm emission 540 (20) nm; mKate2 excitation 588 (9) nm emission 633 (20) nm. Between measurements, the well plate was kept in a shaking incubator (100 rpm) at 37 °C and individual wells were exposed to the appropriate light conditions through the custom-made light-addressable multiwell plate (Supplemental Figure 3).

Data were analysed through custom python script. Blank absorbance of the medium and autofluorescence of bacteria DHL708 were removed. Absorbance was then used to normalize fluorescence with respect to cell count.

Plate reader experiments to detect constructs expression range

Samples were grown from glycerol stocks overnight as in the previous protocol. Cells were refreshed in imaging medium plus antibiotics and OD600 was set to 0.002. Samples were transferred to a 96-well plate and placed in a shaking incubator at 37°C. There, individual wells were exposed to homogeneous levels of red light and a gradient of green light with the custom device in Supplemental Figure 3. Absorbance and sfGFP fluorescence were measured every hour by temporarily moving the plate from the incubator to the plate reader. Data were analysed through custom python script. Protein production rate was calculated as the time derivative of sfGFP concentration, divided by OD in a selected interval of exponential growth.

Mother machine fabrication

Master molds with mother machine features were fabricated using a hybrid technique involving standard soft-lithography and two-photon polymerization [38]. Firstly, the feeding channel (50 μm -width, 15 μm -height) was fabricated using standard protocols of soft-lithography. Precisely, a SU-8 layer of 15 μm was fabricated by spinning SU-8 2015 onto a soda-lime coverglass (3000 rpm -30 s, Laurell WS-650Mz-23NPPB). Strong adhesion of the SU8 to the carrier cover-glass was ensured by three layers of OmniCoat adhesion promoter (MicroChem Corp). The coverglass was soft-baked, and a microfluidic mask with a pattern of 50 μm -wide channels was projected onto the photoresist (UV KUB 2, 400 mJ - 40 s at 25% of maximum power). The coverglass was soft-baked, post-baked, and developed according to the protocol provided by MicroChem Corp. In addition, the sample was hard-baked for 30 min at 170 °C. A second layer of 20 μm was fabricated on top of the feeding channel by spinning SU-8 2015 at 2000 rpm, followed by a soft-baking of 2 hours at 95 °C. Then, the microfabrication of the micron-size channels was carried out by a custom-built two-photon polymerization setup [39]. The refractive index contrast between cured and uncured SU-8 was used to identify the edge of the largest channel, where a comb of micrometer-sized channels was made by direct laser writing. The whole coverglass was again baked at 95 °C and rinsed with PGMEA and IPA. Finally, the master mold was silanized to prevent the PDMS from adhering to the master. PDMS was prepared from a Sylgard 184 Silicone Elastomer kit: polymer base and curing agent were mixed in a 10:1 ratio, and air bubbles were removed from the mixture by centrifugation. The degassed mixture was then poured over the master, and the devices were cured for about 1.5 hours at 90 °C. PDMS chips were peeled from the master mold and bonded to glass by oxygen plasma treatment (15 seconds at 100 watts) and then baked for 10 minutes at 120 °C.

Mother machine experiments

Cells were grown from a glycerol stock for 16 hours in LB with appropriate antibiotics, in a 50 mL centrifuge tube exposed to saturating 660 nm red light. Bacteria were refreshed 1:100 in imaging medium under red light until they reached an OD of 0.1 (around 4 hours). 1 mL of culture was centrifuged in an Eppendorf miniSpin Plus centrifuge at 1000 rcf for 5 minutes and concentrated 50x. 20 μL of concentrated bacteria were loaded in the mothermachine's flow channel with a pipette. Bacteria were loaded in the growth channels, by centrifugation of the mothermachine chip for 7 minutes at 1300 rcf (with soft ramp acceleration) in an Eppendorf Centrifuge 5430R using the Combislide adapter. The flow channel of the mothermachine was then washed for approximately 5 minutes with LB with antibiotics and 0.1% BSA at a constant flow of 50 $\mu\text{L}/\text{min}$, to carry away excess bacteria. For the whole duration of the experiments, the mothermachine was perfused with LB with antibiotics and 0.1% BSA at a constant rate of 5.5 $\mu\text{L}/\text{min}$.

Microscopy

Phase contrast and epi-fluorescence imaging were performed using a custom-built optical microscope equipped with a 100 magnification objective (Nikon MRH11902; NA=1.3) and a high-sensitivity CMOS camera (Hamamatsu Orca-Flash 4.0 V3) (see Supplemental Fig. 7). For phase

contrast imaging, a deep red LED (Thorlabs M730L4) with low inhibition of the optogenetic expression system was used. Light control was achieved by directing light through a 10:90 beam splitter (R:T, Thorlabs BSN10R) positioned under the objective. Green and red light stimuli were provided by two LEDs (Thorlabs M530L4, Thorlabs M660L4, respectively) coupled through a dichroic mirror (Thorlabs DMLP567R) and passing through a long-pass filter at 550 nm (Thorlabs FELH0550). Epi-fluorescence imaging was conducted using a blue LED (Thorlabs M455L4) and a filter set for the CFP (Chroma 49001-ET-ECFP). To enable imaging in both phase contrast and epi-fluorescence, a motorized filter wheel (Thorlabs FW102C) was used to switch between the fluorescence filter set and a long-pass filter at 700 nm (Thorlabs FWLH0700). To maintain sample focus throughout the entire acquisition process, a custom script was developed. This script scans the sample in height finally moves to the z-plane where the image of a specific fixed structure in the chip has maximum edge enhancement (calculated with the Sobel function). To avoid drift on the x-y plane, we correlate the acquired image of the fixed structure with one acquired at the beginning of the experiment and compute the shift on the x-y plane. The scanning process was performed using a motorized vertical lift stage (Zaber X-VSR-E) and a motorized microscope stage (Zaber X-ASR100B120B-SE03D12).

Segmentation of mother machine data

Phase contrast images were taken every 3 min and fluorescence images every 9 min. Phase contrast images were analyzed, to identify the single feeding channels of the microfluidic chip. Single cells were segmented using the pretrained neural network model Cellpose 2.0. We further trained the model with our own data to increase the accuracy of segmentation [40]. The masks obtained after segmentation were used to measure the total fluorescence of the mother cell in all feeding channels of the mother machine as a function of time.

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Author contributions

R.D.L. conceived the project. M.C.C. designed and constructed all plasmids and strains, with G.F. and F.L. collaborating to plasmid design.

N.P. developed mothermachine chips. G.F. and R.D.L. designed optics. M.C.C., G.F., and F.L. conducted experiments and analysed the data, with N.P. contributing specifically to the mothermachine's experiments and analysis. All the experiments have been performed under the supervision of G.F. and R.D.L. M.C.C., F.L., R.D.L. carried out simulations. All authors contributed to the writing of the manuscript.

Competing interests

The authors declare no competing interests.

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Competing interests

The authors declare no competing interests.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper and the Supplementary Materials.

Supplementary Materials

Strains

Strain	Genotype	Source	Parent
DHL708	MC4100 $\Delta clpPX$	[8]	
MCC0234	DHL708 $\Delta attB :: lacI34$ $P_{cpcG2-175} BBa_B0034 lacI (kan)$	this work	DHL708
MCC0233	DHL708 $\Delta attB :: lacI33$ $P_{cpcG2-175} BBa_B0033 lacI (kan)$	this work	DHL708
MCC0034	DHL708 $\Delta attB :: gfp34$ $P_{cpcG2-175} BBa_B0034 sf gfp (kan)$	this work	DHL708
MCC0033	DHL708 $\Delta attB :: gfp33$ $P_{cpcG2-175} BBa_B0033 sf gfp (kan)$	this work	DHL708

Table 1

***Escherichia coli* strains used in this work.**

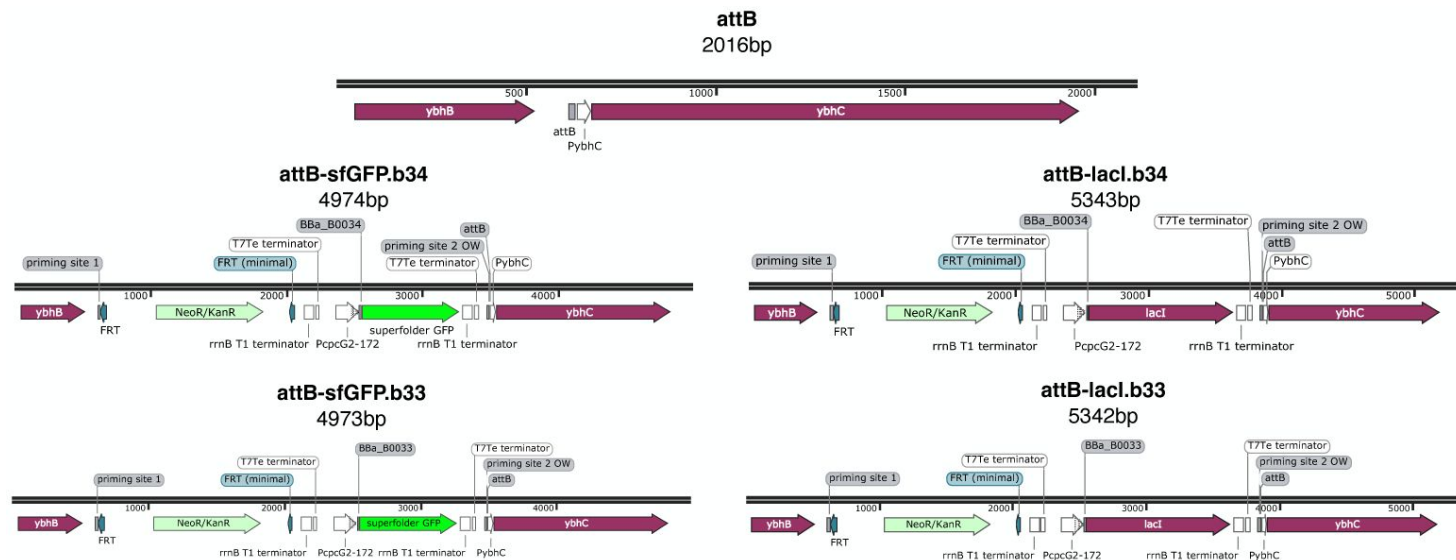


Fig. 7

Maps of the original *attB* site and genome insertions.

Genome sites correspond to *E. coli* strains: MCC0034 top left, MCC0033 bottom left, MCC0234 top right, MCC0233 bottom right. All maps are created with SnapGene.

Plasmids

Plasmid	Description	Source	Parent
pLPT234	core repressilator components and reporters	[9]	
pLPT145	repressors' sponge	[8]	
pNO286-3	light sensor and PCB enzymes	[32]	
pSR58.6	CcaR, light-driven sfGFP production (RBS BBa_B0034)	[31]	
pSR58.b33	CcaR, light-driven sfGFP production (RBS BBa_B0033)	this work	pSR58.6
pSpongeROG.b34	light-driven LacI production (RBS BBa_B0034), repressors's sponge, CcaR	this work	pSR58.6 (backbone), pLPT145 (sponge), pLPT234 (<i>lacI</i>)
pSpongeROG.b33	light-driven LacI production (RBS BBa_B0033), repressors's sponge, CcaR	this work	pSR58.6 (backbone), pLPT145 (sponge), pLPT234 (<i>lacI</i>)
pSpongeROG0	repressors's sponge, CcaR	this work	pSR58.6 (backbone), pLPT145 (sponge)
pKD46-sfGFP.b34	genome insertion of light-driven sfGFP cassette	this work	pKD46 (backbone), pSR58.6
pKD46-sfGFP.b33	genome insertion of light-driven sfGFP cassette	this work	pKD46 (backbone), pSR58.b33
pKD46-lacI.b34	genome insertion of light-driven LacI cassette	this work	pKD46 (backbone), pSpongeROG.b34
pKD46-lacI.b33	genome insertion of light-driven LacI cassette	this work	pKD46 (backbone), pSpongeROG.b33

Table 2

Plasmids

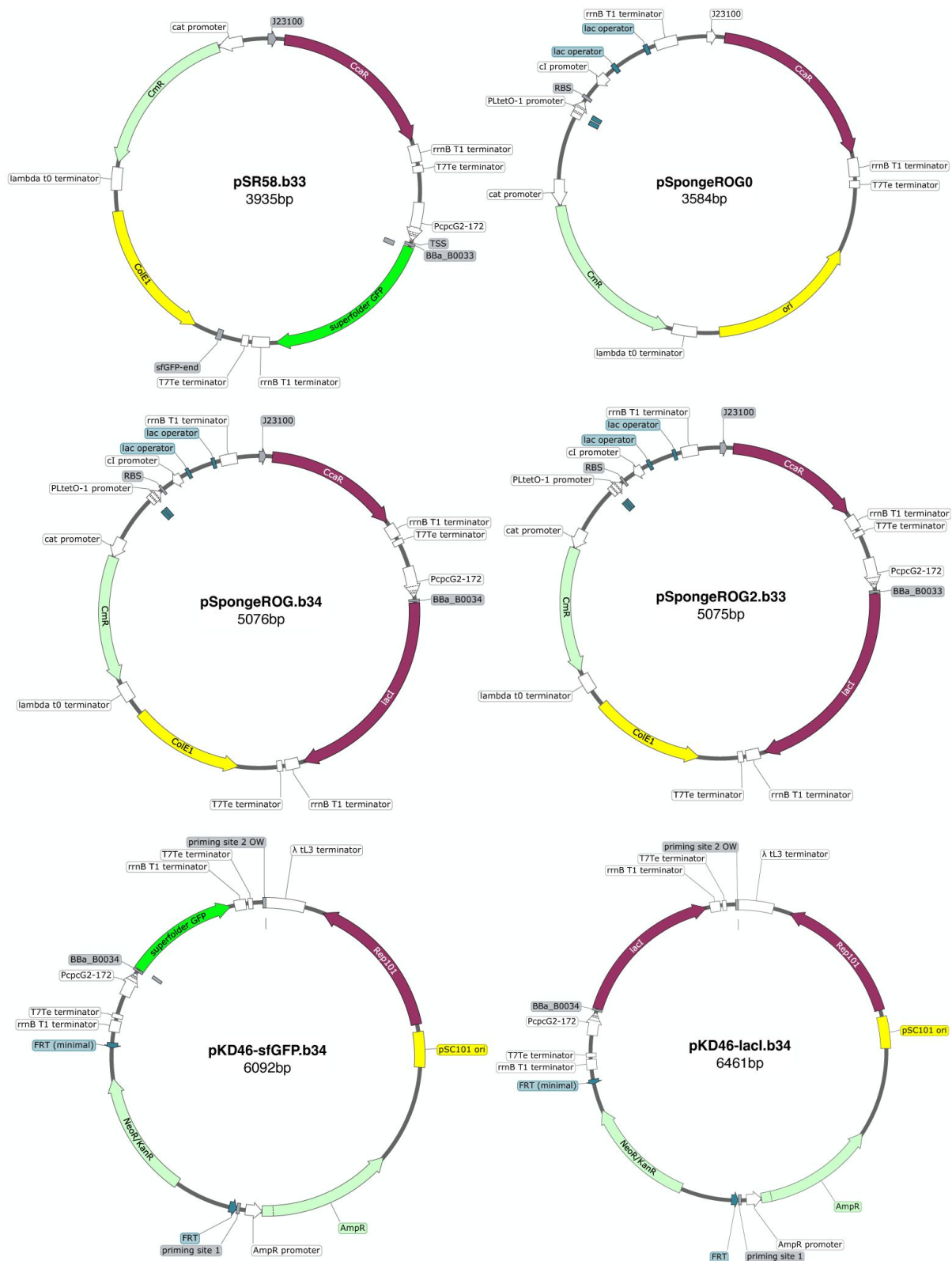


Fig. 8

Plasmid maps.

All maps are created with SnapGene.

Antibiotic concentrations

Antibiotic	working concentration
Streptomycin	50 $\mu\text{g/ml}$
Ampicillin	100 $\mu\text{g/ml}$
Kanamycin	50 $\mu\text{g/ml}$
Chloramphenicol	20 $\mu\text{g/ml}$
Spectinomycin	100 $\mu\text{g/ml}$

Table 3

Antibiotic concentrations

Custom-made light-addressable multiwell plate

The custom-made light-addressable multiwell plate was made using an Adafruit NeoPixel NeoMatrix 8x8, a matrix of 64 RGB LEDs, attached to the bottom of a Greiner 96 well plate with black wells and removed bottoms. The matrix was placed so that each LED was directly under an individual well. An opaque foam layer was cut in order to accommodate LEDs. It was used to adjust the matrix to the plate and to reduce well-to-well light contamination. An additional plate was fixed on top of this structure to decrease light intensity on samples by increasing the distance between LEDs and samples. Opaque foam layers were cut in correspondence with the wells and attached above and below this plate, again to avoid well-to-well light contamination. The construct was fixed to a shaking incubator. A 96 well plate could be placed on top of it and removed for data acquisition. A darkened lid was placed on the samples' plate to block environment light.

The LEDs matrix was controlled with an Arduino microcontroller. To ensure correct timing of inputs, we employed an AZDelivery Real Time Clock module.

LEDs intensity on samples was measured with Thorlabs Standard Photodiode Power Sensor S121C.

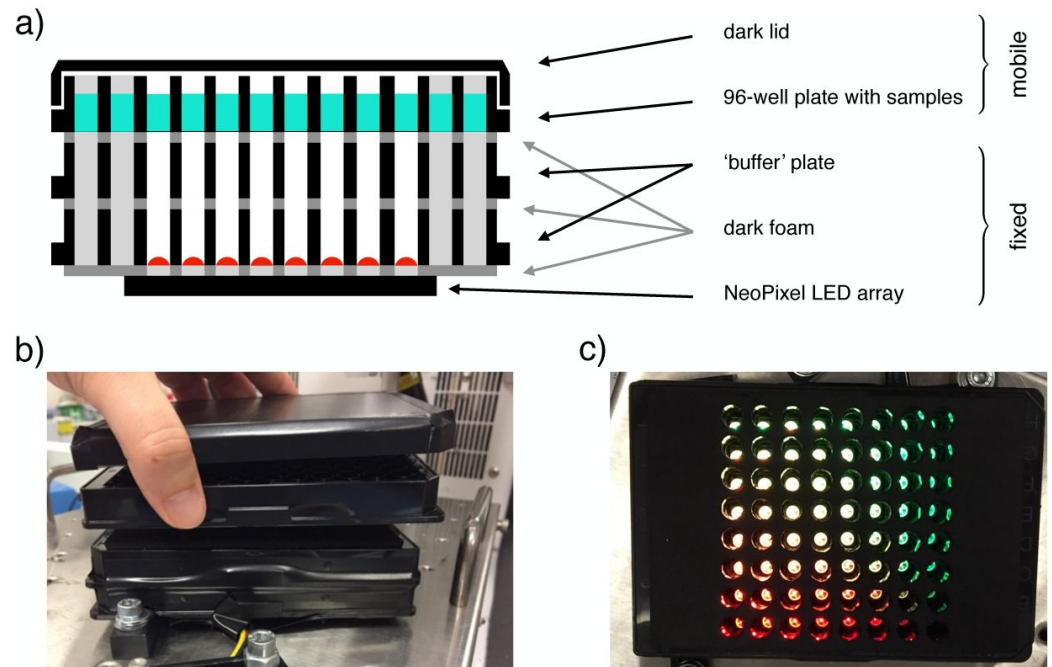


Fig. 9

The custom-made light-addressable multiwell plate

a) Scheme of a longitudinal section of the device, with key components highlighted. **b)** Frontal picture of the custom-made light-addressable multiwell plate placed in a shaking incubator. The sample plate and dark lid are partially lifted. **c)** Top-down picture of the device. Dark lid and sample plate are removed and LEDs are on.

sfGFP production per light intensity

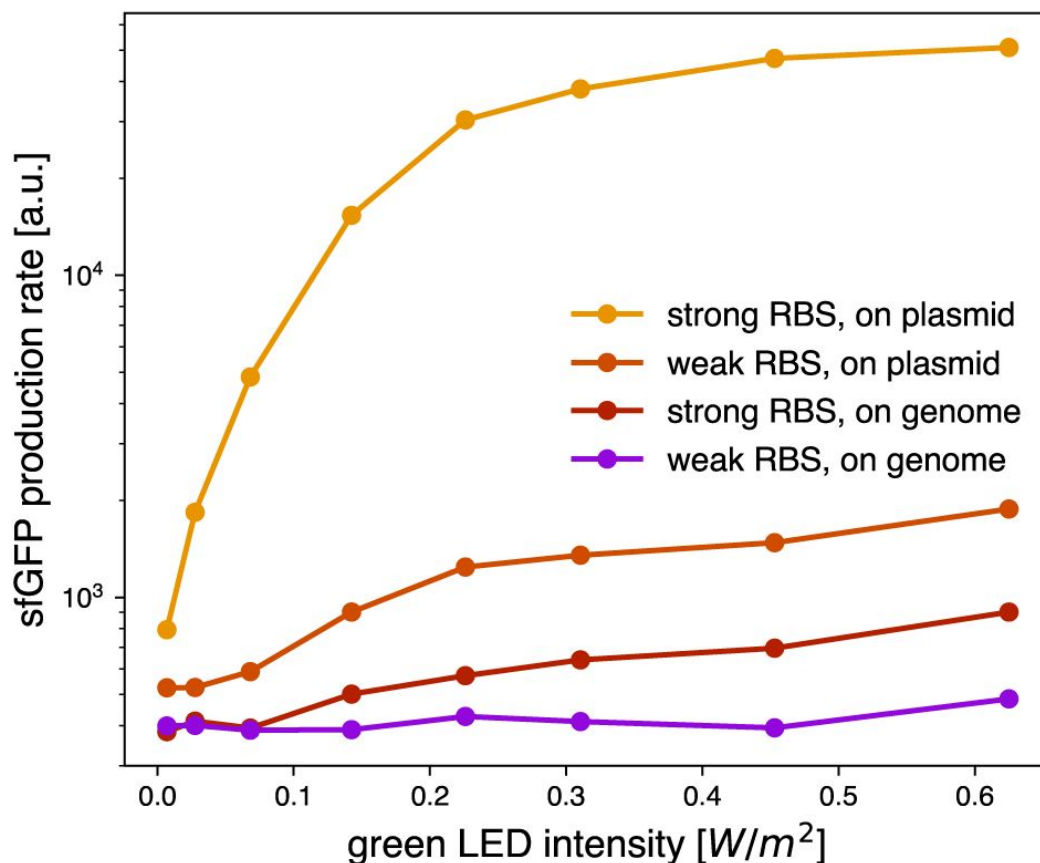


Fig. 10

sfGFP production rate per light intensity.

All samples are DHL708 *E. coli* strain with pNO286-3 plasmid, added with sfGFP light-driven production construct as shown in Fig. 3 of the paper. While being exposed to different green light intensities, all samples were also constantly exposed to $0.74W/m^2$ red light. Dots are the mean values of experiments repeated on 3 different days. Lines are interpolation between experimental values.

Analytical solution of repressilator dynamics in the digital approximation

In the digital approximation Hill functions in Eq. 1 in the main text are replaced by Θ functions and equations become piecewise linear first order. Oscillations are therefore built by joining together rising (to steady value β/α) or decaying (to 0) exponentials with rate α . Calling with M and m respectively the maximum and minimum values in the oscillations. t is the interval between

two maxima of two different proteins and δ is the time interval to rise from the minimum m to 1 (Fig. 11). All concentrations are measured in units of K so 1 represents the activation threshold. We can write the following four equations in the unknowns m , M , t , δ :

$$Me^{-(t-\delta)} = 1 \quad (3)$$

$$e^{-(t+\delta)} = m \quad (4)$$

$$\beta + (1 - \beta)e^{-t} = M \quad (5)$$

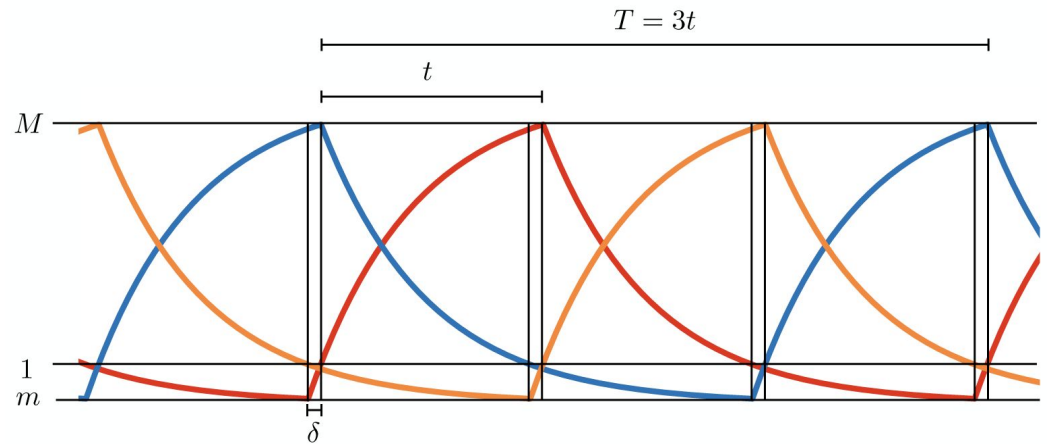


Fig. 11

Simulations of the digital model. M and m , respectively, are the maximum and minimum values in the oscillations and δ is the time interval to rise from the minimum m to 1 (threshold value in K units).

$$\beta + (m - \beta)e^{-\delta} = M \quad (6)$$

Where, for simplicity of notation, we measure time in units of α^{-1} . From Eq.(1) and (2) we find:

and

$$e^{-2t} = m/M \quad (7)$$

$$e^{-2\delta} = Mm \quad (8)$$

which substituted in (3) and (4) give:

$$\beta + (1 - \beta)\sqrt{m/M} = M \quad (9)$$

$$\beta + (m - \beta)\sqrt{Mm} = 1 \quad (10)$$

The two equations above can be solved numerically to get M and m once β is known. However if, as in our case, $\beta \gg 1$ we can find the approximate solutions:

$$M = \beta, \quad m = 1/\beta \quad (11)$$

Substituting back in (5) we get

$$t = \log \beta \quad (12)$$

and thus for the entire period

$$T = 3t = 3 \log \beta \quad (13)$$

Finally reintroducing the time scale α we can write for the period

$$T = \frac{3}{\alpha} \log \frac{\beta}{\alpha} \quad (14)$$

Microscope setup

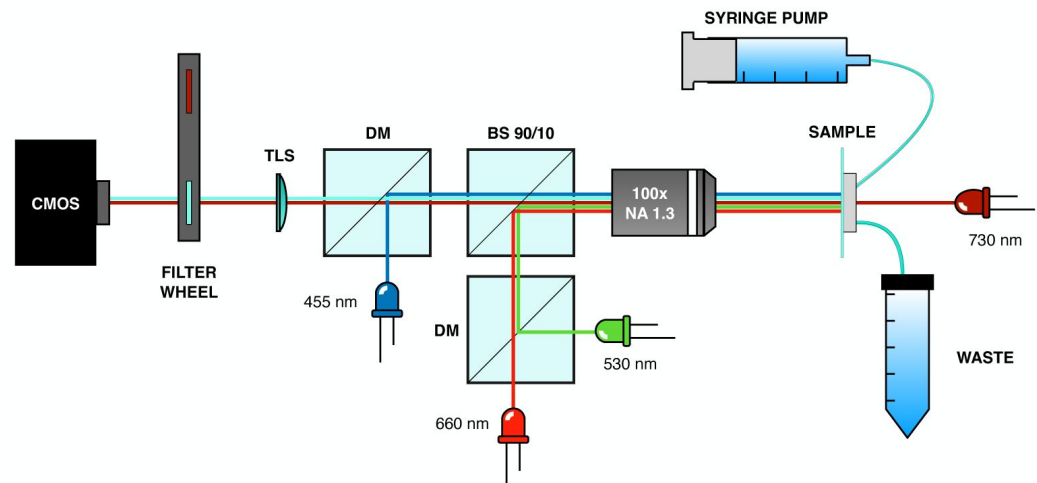


Fig. 12

Microscope setup used for mothermachine experiments

Description of Supplemental Videos

The snapshots of CFP fluorescence were acquired every 9 minutes and the videos are played at 32 frames/s. The presence of a green square over the image indicates that the bacteria are exposed to green light at that time point. The fluorescence of each mother cell and the mean fluorescence of all mother cells are plotted as a function of time in **Fig. 4b** (Suppl. Video 1) and **Fig. 5d** (Suppl. Video 2).

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Reviewer #1 (Public Review):

Summary:

The "optorepressilator", an optically controllable genetic oscillator based on the famous *E. coli* 3-repressor (LacI, TetR, CI) oscillator "repressilator", was developed. An individual repressilator shows a stable oscillation of the protein levels with a relatively long period that extends a few doubling times of *E. coli*, but when many cells oscillate, their phases tend to desynchronize. The authors introduced an additional optically controllable promoter through a conformational change of CcaS protein and let it control how much additional CI is produced. By tightly controlling the leak from the added promoter, the authors successfully kept the original repressilator oscillation when the added promoter was not activated. In contrast, the oscillation was stopped by expressing the additional CI. Using this system, the authors showed that it is possible to synchronise the phase of the oscillation, especially when the activation happens as a short pulse at the right phase of the repressilator oscillation. The authors further show that, by changing the frequency of the short pulses, the repressilator was entrained to various ratios to the pulse period, and the author could reconstruct the so-called "Arnold tongues", the signature of entrainment of the nonlinear oscillator to externally

added periodic perturbation. The behaviour is consistent with the simplified mathematical model that simulates the protein concentration using ordinary differential equations.

Strengths:

Optical control of the oscillation of the protein clock is a powerful and clean tool for studying the synthetic oscillator's response to perturbation in a well-controlled and tunable manner. The article utilizes the plate reader setup for population average measurements and the mother machine setup for single-cell measurements, and they compensate nicely to acquire necessary information.

Weaknesses:

The current paper added the optogenetically controlled perturbation to control the phase of oscillation and entrainment, but there are a few other works that add external perturbation to a collection of cells that individually oscillate to study phase shift and/or entrainment. The current paper lacks discussion about the pros and cons of the current system compared to previously analyzed systems.

<https://doi.org/10.7554/eLife.97754.1.sa1>

Reviewer #2 (Public Review):

Summary:

In this manuscript by Cannarsa et. al., the authors describe the engineering of a light-entrainable synthetic biological oscillator in bacteria. It is based on an upgraded version of one of the first synthetic circuits to be constructed, the repressilator. The authors sought to make this oscillator entrainable by an external forcing signal, analogous to the way natural biological oscillators (like the circadian clock) are synchronized. They reasoned that an optogenetic system would provide a convenient and flexible means of manipulation. To this end, the authors exploited the CcaS-CcaA light-switchable system, which allows activation and deactivation of transcription by green and red light, respectively. They used this system to make the expression of one of the repressilator's transcription factors (lacI) light-controlled, from a construct separated from the main repressilator plasmid. This way, under red light the oscillator runs freely, but exposure to green light causes overexpression of the lacI, pushing the system into a specific state. Consequently, returning to red light will restore the oscillations from the same phase in all cells, effectively synchronizing the cell population.

After demonstrating the functionality of the basic concept, the authors combined modeling and experiments to show how periodic exposure to green light enables efficient entrainment, and how the frequency of the forcing signal affects the oscillatory behavior (detuning).

This work provides an important demonstration of engineering tunability into a foundational genetic circuit, expands the synthetic biology toolbox, and provides a platform to address critical questions about synchronization in biological oscillators. Due to the flexibility of the experimental system, it is also expected to provide a fertile ground for future testing of theoretical predictions regarding non-linear oscillators.

Strengths:

* The study provides a simple and elegant mechanism for the entrainment of a synthetic oscillator. The design relies on optogenetic proteins, which enable efficient experimentation compared to alternative approaches (like using chemical inducers). This way, a static culture (without microfluidics or change of growth media) can be easily exposed to flexible temporal sequences of the zeitgeber, and continuously measured through time.

* The study makes use of both plate-reader-based population-level readout and mother-machine single-cell measurements. Synchronization through entrainment is a single cell level phenomenon, but with a clear population-level manifestation. Thus, this experimental approach combination provides a strong validation to their system. At the same time, differences between the readout from the two systems have emerged, and provided a further opportunity for model refinement and testing.

* The authors correctly identified the main optimization goal, namely the effective leakiness of their construct even under red light. Then, they successfully overcame this issue using synthetic biology approaches.

* The work is supported by a simplified model of the repressilator, which provides a convenient analytical and numerical means to draw testable predictions. The model predictions are well aligned with the experimental evidence.

Weaknesses:

* Even after optimizing the expression level of the light-sensitive gene, the system is very sensitive, i.e., a very short exposure is sufficient to elicit the strongest entrainment. This limited dynamic range might hamper some model testing and future usage.

* As a result of the previous point, the system is entrained by transiently "breaking" the oscillator: each pulse of green light represents a Hopf bifurcation into a single attractor. It means that the system cannot oscillate in constant green light. In comparison, this is generally not the case for natural zeitgebers like light and temperature for the circadian rhythms. Extreme values might prevent oscillations (not necessarily due to breaking the core oscillator), but usually, free running is possible in a wide range of constant conditions. In some cases, the free-running period length will vary as a function of the constant value.

While the approach presented in this manuscript is valid, a comprehensive analysis of more subtle modes of repressilator entrainment could also be of value.

* The entire work makes use of a single intensity and single duration of the green pulse to force entrainment. While the model has clear predictions for how those modalities should affect entrainment, none of the experiments attempted to validate those predictions.

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