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The pedestrian watchmaker: Genetic clocks from engineered oscillators

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Abstract

The crucial role of time-keeping has required organisms to develop sophisticated regulatory networks to ensure the reliable propagation of periodic behavior. These biological clocks have long been a focus of research; however, a clear understanding of how they maintain oscillations in the face of unpredictable environments and the inherent noise of biological systems remains elusive. Here, we review the current understanding of circadian oscillations using *Drosophila melanogaster* as a typical example and discuss the utility of an alternative synthetic biology approach to studying these highly intricate systems.

Keywords

Synthetic biology; Circadian clock; Genetic oscillator

1. Introduction

Richard Dawkins coined the phrase “The Blind Watchmaker” in order to illustrate how Darwinian evolution refutes the “watchmaker analogy” [1]. The watchmaker analogy has often been used to authoritatively assert that observed complexity necessarily implies design by an omnipotent engineer [2]. While such logic is oddly absurd in the context of evolution, less supernatural engineers and scientists have taken up the task of designing and constructing biological complexity at the genetic level. This design process was the original defining goal of the new discipline of synthetic biology [3–6], which has recently evolved to encompass a large swath of academic and industrial pursuits that now includes all of genetic engineering.

While the watchmaker analogy does not provide insight into the methodology that was employed by the Grand Bioengineer, the modern synthetic biology approach involves a multi-disciplinary milieu [3,4,7–12]. First, genetic wiring diagrams are translated into equations that can be analyzed [13–15]. This step is analogous to the electrical engineer’s use of Ohm’s and Kirchhoff’s laws for modeling electronic circuits. Next, tools from applied math and computer science are used to analyze the model in order to extract the “design criteria” for a desired output [4–6,16–18]. Then modern recombinant DNA techniques are

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used to construct gene-regulatory networks in living cells according to the design specifications. Typically, this step involves the use of fluorescent reporter genes that allow for optical measurements [19,20]. Lastly, micro- and nano-technologies are developed to acquire the precise single-cell measurements that are needed for comparison with model predictions and design refinement [21–27].

The forward-engineering paradigm provides an attractive organizational framework for conducting research in the face of the enormous complexity associated with gene regulation. However, while this approach has proven to be particularly well-suited for popular consumption, there is a crucial yet often deemphasized difference between the design of electronic and genetic circuits. The description of electronic circuits follows from physical laws (e.g. Maxwell's equations) describing the connection of engineered components in a controlled physical environment. Genetic circuits, on the other hand, consist of evolved components that reside in the highly complex biological environment of the cell. Given this complexity, it would be exceedingly optimistic to expect that mesoscopic laws describing gene regulation will elegantly arise from the underlying physical chemistry (in this century). In this sense, perhaps an apt analogy is classical 19th-century thermodynamics, which was based on empirical foundations that made it extremely useful since it was (and is) impervious to any underlying microscropic theory. Likewise, our ability to understand gene regulation and construct genetic circuits according to "design specs" rests on the *empirical* determination of the mesoscopic laws governing their behavior. This underscores the importance of the last step in the forward-engineering protocol, which involves the development of new measurement technologies for careful comparison between experiment and model.

Given that time-keeping is fundamental to life, it is not surprising that watches (or clocks) are of broad interest. In addition to lofty philosophical analogies, oscillatory clocks are of course fundamental to science and engineering. At the level of genomics, one of the most widely studied and enigmatic network phenomena is the oscillatory behavior of "biological clocks." Even after years of research involving a broad range of approaches, biological clocks continue to be a focus of research, because new studies using novel techniques continue to unveil additional cyclic transcripts, new processes under clock control, and key regulators of clock-controlled processes. Organisms from bacteria and fungi to plants and animals have evolved a wide variety of timing mechanisms to govern periodic behavior, and it is believed that these clock networks may have evolved independently [28]. While the mechanisms that lead to oscillatory behavior vary in complexity and detail, certain "design principles" are common to their underlying gene-regulatory networks.

A ubiquitous feature of biological clocks is the presence of a core autoregulatory gene network. In particular, a common motif is the employment of interlocked feedback loops, involving both positive and negative autoregulation of a small number of core clock genes [29–31]. This motif of coupled positive and negative feedback loops is common in circadian clock networks as well as in the segmentation clock networks that regulate pattern formation in the early stages of development [32,33]. Similar regulatory motifs form the core modules that control many important biological processes in eukaryotic systems, such as inflammation mediated by the NF-κB network [34], tumor suppression mediated by the p53 network [35], and genetic competence [36]. All of these networks are capable of generating self-sustained oscillatory behavior, which suggests that this dual-feedback motif is an attractive module for designing and constructing a genetic clock using synthetic biology.

The complex dynamic behavior of biological clocks makes them a particularly attractive target for the combined approach of synthetic biology and computational analysis. However, despite the wealth of information accumulated on natural biological clocks, it remains a

challenge to engineer a synthetic system that is capable of mimicking their behavior. The difficulty of emulating native behavior indicates that important pieces of pertinent information are still missing. The parameters that determine network dynamics are difficult to measure and impossible to ascertain from static network diagrams. Therefore, forward engineering can be a highly informative approach to studying the dynamics that arise from complex network topologies, as step-by-step reconstruction can contribute invaluable information about key network properties.

A common two-element motif consisting of coupled positive and negative feedback, in which a promoter drives the production of both its own activator and repressor, is theoretically capable of exhibiting periodic behavior [37]. However, in order to achieve sustained oscillations, certain design principles have to be implemented based on lessons learned from natural clock networks. The circadian oscillator of *Drosophila melanogaster* has been characterized in great depth and has proven to be a paradigm model for higher mammalian clocks [30,38]. In the next section we review what is known about the core of the *Drosophila* clock network and what lessons we can learn about the fundamental requirements for a robust oscillatory system. Then, we describe our recently developed synthetic oscillator which implements these lessons in a living organism. Finally, we discuss how this synthetic biology approach can help reveal design principles that are key to driving and regulating dynamic behavior but that may be masked by the complexity of native networks.

2. The *Drosophila* circadian oscillator

Circadian rhythms have evolved in many organisms as a means to keep time, conferring the ability to anticipate the day–night cycle and adapt behavior to meet the changing conditions throughout the 24 h period. Circadian networks are inherently complex at all levels, from the underlying molecular mechanism for generating the rhythm to the intricate interactions that enable the central oscillator to drive circadian behaviors. In order to ascertain the dynamics of the fundamental core clock mechanism, much of the research to date has focused on a few genetically tractable model organisms. As research methods have become more sophisticated and large-scale genomic analysis has become a reliable tool for comparing networks across species, it has become clear that circadian rhythms in flies and mammals share similar network motifs and highly conserved key clock components (Fig. 1) [38,39]. Therefore, because the genetics of circadian rhythms in *Drosophila* has elucidated the working principles of the circadian clock, we choose to focus on this organism for the purpose of paring down the core clock mechanism to a few basic design principles that are required for generating oscillations.

The *per* gene of *Drosophila* was one of the first genes to be characterized that has a direct role in maintaining a circadian rhythm. Early phenotype-based screens of mutant flies led to the discovery of the role of *per* in regulating the length of the period [40]. Further analysis of the network led to the discovery of a second gene, *tim*, that was also required for the circadian rhythmicity of *Drosophila* [41]. These two genes were identified to form the core of a transcriptional feedback loop which drives the periodic nature of the network (Fig. 1) [28,30]. Interestingly, this core feedback mechanism is conserved between flies and mammals, and there are mammalian orthologs of both *per* and *tim* [30,38].

One common theme at the core of circadian networks is the coexistence of coupled autoregulatory loops involving positive and negative feedback elements [42]. In the case of *Drosophila*, the two genes *clk* and *cyc*, which also have mammalian orthologs, participate both in positive and negative feedback loops. The CLK and CYC proteins heterodimerize, bind to the PER and TIM promoters, and positively regulate their expression [43]. It is this

CLK/CYC complex activity that is inhibited by the PER/TIM complex to complete the core negative feedback loop. Additionally, there is evidence that the PER/TIM complex also acts to positively regulate the transcription of *clk* gene, thereby completing a positive feedback loop [29,44]. Both of these feedback loops operate with significant time delays of up to several hours due to multi-step transcription and translation processes [29].

While the *per/tim* and *clk/cyc* genes form the core of the transcriptional feedback loop, there are several other genes that contribute additional regulatory functions and refine the periodic behavior of the network. In the *Drosophila* circadian clock, additional time delay arises in part due to the destabilization of PER and TIM. The constitutively expressed DBT protein is responsible for a delay in the production of functional PER protein, by regulating the phosphorylation and stability of PER and thus the level of accumulation of PER monomers [45,46]. This promotes a delay between *per/tim* transcription and PER/TIM complex function. SGG is another constitutive kinase, which is involved in TIM phosphorylation and nuclear entry of the PER/TIM complex [47].

Finally, a critical aspect in sustaining a periodic rhythm is the controlled degradation of key proteins. Studies investigating gene dosage revealed that tight regulation of the steady state expression levels of clock components is important to generate and sustain a precise rhythm [48]. The PER protein is an obvious target for tightly controlled degradation, due to its important role in maintaining the circadian rhythm. The degradation of PER is believed to be controlled by the SLMB protein, which targets phosphorylated PER for degradation in the proteasome [49,50]. DBT promotes the progressive phosphorylation of PER, leading to the rapid degradation of hyperphosphorylated isoforms by the ubiquitin–protea-some pathway.

Model circadian networks such as the one found in *Drosophila* have generated a good understanding of the core clock proteins and their roles in maintaining the daily rhythm (Fig. 1). These studies have revealed important insights into the molecular biology and mechanisms that underlay circadian oscillator function, not just in the model systems but in all organisms. However, the complexity of the networks and their intertwinement with other cellular processes has made it difficult to develop maps of all network components and to deduce how components interact to contribute to the overall function of the time-keeping mechanism. The core module of coupled feedback is wrapped in a complex network involving many layers of regulation. However, knockout studies of circadian systems as well as computational analysis have suggested that a simplified two-component module is theoretically capable of sustaining periodic behavior on its own [37,40,51–53]. By decoupling a relatively simple module from its complex biological setting, we may be able to systematically explore a design principle that has evolved to regulate periodic cellular behavior and use this simplified system to determine how additional components add complexity, regularity, and robustness to a clock's function.

3. Basic science through engineering: synthetic oscillators

The possibility of a minimal core network driving robust cellular behavior has inspired the development of an alternative approach to the study of gene-regulatory networks: create the network, beginning with a one or two-component system and then rebuild the network from the bottom up. In this way, we can gradually assemble increasingly complex systems that mimic the native network, while maintaining at each stage the ability to model and test the network in a tractable experimental system.

There have been several successful attempts at developing a synthetic oscillatory network controlled at the gene regulation level [5,54–56]. These networks involved only two- or three-components, and mathematical modeling was instrumental in the process of designing

and analyzing the network structure and revealing the mechanism behind their ability to exhibit periodic behavior. In *Escherichia coli*, the *repressilator* [5] consisted of a ring architecture of cyclic repression that was capable of generating sustained oscillations in a subset of the cells that were examined, while a two-component feedback-based circuit [54] was shown to generate damped oscillations. A synthetic mammalian oscillator based on an autoregulated sense–antisense transcription control circuit yielded self-sustained and tunable oscillatory gene expression in a fraction of the cells observed [55].

These examples represent progress in implementing an engineering-based approach to the study of gene networks, in which computational modeling is used to guide the design of novel networks and accurately predict their dynamic behavior. However, the lack of robustness in each of these networks demonstrates the need to focus on a network architecture that more closely mimics native networks. In this way, we can hope to elucidate the properties that enable organisms to maintain stable oscillations in the face inherently noisy and ever-changing microenvironments.

Recently, we designed and constructed a novel two-component oscillator, based on principles observed to be critical for the core of a circadian clock network (Fig. 2) [57]. The design of the oscillator was based on our earlier work involving coupled positive and negative feedback loops [37]. Computational modeling was used to develop design criteria for achieving oscillations in this system. These criteria included an effective separation of timescales between the positive and negative components, strong activation and tight repression of the promoter, and fast degradation rates for the proteins. Importantly, the design also implied that the components should be carefully tuned in order to achieve oscillations; i.e. most parameter values would not lead to oscillations in this design.

The construction of the synthetic gene oscillator employed a hybrid promoter ($P_{1ac/ara-1}$; [58]), which is composed of the activation operator site from the *araBAD* promoter placed in its normal location relative to the transcription start site, and repression operator sites from the *lacZYA* promoter placed both upstream and immediately downstream of the transcription start site. Negative and positive feedback were achieved by putting LacI and AraC, respectively, under control of $P_{1ac/ara-1}$. The promoter is activated by the AraC protein in the presence of arabinose and repressed by the LacI protein in the absence of IPTG, so that the anticipated necessity of tuning was easily implemented through chemical inducers and without the need for further genetic modifications. The separation of timescales was implemented by placing the activator on a high-copy ColE1 plasmid, while the repressive element was placed on a medium copy p15 plasmid. Finally, fast degradation was achieved using ssrA-tagged proteins.

While the careful design and construction of novel circuits is critical to the forward-engineering approach, another essential aspect is the ability to characterize the behavior of these new networks inside living cells. This requires the acquisition of finely-sampled time course data of individual cells that can be compared to simulations for the refinement of the underlying theory. Other techniques, such as flow cytometry and fluorometry, have long been used to take static, population measurements of fluorescent reporters in living cells, but these techniques do not allow for the careful characterization of dynamic behavior in single cells over time. For this reason, the development of microfluidic devices to enhance the acquisition of time course microscopy data has been a focus of several research groups over the recent years [21–27].

For the purpose of characterizing a highly dynamic network under multiple inducer conditions at the single-cell level, we developed a microfluidic chip tailored for the long-term imaging of *E. coli* [24]. The critical design feature for this device was a shallow

imaging chamber of only 1 μm in height, in order to constrain a large population of exponentially growing cells to a monolayer. The chip was designed to supply a continuous flow of fresh media through the imaging chamber, fast enough to provide sufficient nutrients to the entire population, but slow enough not to disrupt the cells. In this way, hundreds of cells are able to grow and divide for many generations in a single focal plane, providing high quality images of the fluorescent reporter captured every 2 min (Fig. 3). With this high time resolution and the ability to tightly control the temperature and media conditions, we were able to generate data that could be carefully compared to model simulations for many combinations of inducer levels and temperatures.

Given the design of our circuit, we assumed that onerous screening of arabinose and IPTG combinations would be necessary to observe oscillations. However, to our surprise, we observed oscillations in one of the first microscopy experiments. Furthermore, we subsequently observed oscillations over a vast range of IPTG and arabinose values (Fig. 4a–g). After an extensive series of experiments characterizing the period under multiple inducer and growth conditions, we arrived at three principle observations that were difficult to reconcile with the original model design. First, it was difficult to find inducer levels at which the system *did not* oscillate; the network was incredibly robust in generating oscillations. Second, at low arabinose values, the model predicted that the period would increase, while the experiments showed the period to decrease. And third, fitting the experimental data with the original model required suspect parameter values. While it was conceivable that the second and third of these discrepancies were due to some small unknown details, the robust behavior seemed to suggest that there was something fundamentally incorrect in the model. In other words, it became increasingly clear that the observed oscillations did not necessarily validate the model, even though the model had predicted oscillations.

We were able to resolve the discrepancy between model and theory by reevaluating the assumptions that led to the derivation of the model equations. In the original derivation, it was assumed that the coupled positive and negative feedback architecture was central, and that detailed processes such as protein folding, multi-merization, and DNA-binding, were less important and occurred instantaneously. Given the experimental evidence that in bacteria these processes do not take more than a few minutes, this assumption seemed reasonable in the context of oscillations that should occur on timescales of roughly 30 min. However, the relaxation of this assumption and explicit incorporation of intermediate steps led to a very robust oscillator with periods ranging from 15 to 60 min, despite an effective time delay of only a few minutes. In addition, this new model resulted in excellent experimental agreement for the period versus arabinose with very reasonable parameter values.

A natural consequence of the new design principles was that the negative feedback loop with a small delay should in principle be capable of generating oscillations without the positive loop. We predicted that while these oscillations should exist, they would be less regular and that only a small subset of inducer levels would lead to oscillations. These predictions were indeed validated by experiments that showed oscillatory behavior, however considerably less regular and robust than in the coupled positive–negative feedback system (Fig. 4h). Taken together, the experimental and modeling results demonstrated that the core oscillator arose from delayed negative feedback, with the positive loop providing additional robustness, strength, and regularity to oscillations.

Drawing an analogy to integrate-and-fire dynamics in neuroscience, we have coined the term “degrade and fire” oscillations [59] to describe the essence of the dynamics, which can be understood with the aid of Fig. 5 as follows. Effectively, the cascade of reactions leading to the formation of functional transcription factors can be replaced by compound “delayed”

reactions (Fig. 5a). Then the dynamics can be well captured by a delay-differential equation model which permits analytical insight into the nature of the oscillations [59]. According to this model, first the activator "fires," followed shortly by the repressor. Then both promoters are off while both proteins slowly degrade until the repressor degrades completely (Fig. 5b). In this way, the period is largely determined by the decay rate of the repressor, and the amplitude is simply proportional to the period.

4. Lessons and future directions

The successful construction of a synthetic clock network inspired by a design principle commonly found in nature provides a foundation for understanding the fundamentals of native clock networks and for observing how periodic behavior is able to propagate throughout many generations of life. The process of building a synthetic network based on model specifications, followed by the refinement of the model to better describe the experimental observations, led to many interesting discoveries about what made our clock "tick." This, in turn, yielded important insights into the key principles that can drive a biological clock, and these lessons may be applicable to the native networks that inspired our design.

As it has been predicted by the study of native circadian clocks, our synthetic system demonstrates that a two-component network of coupled transcriptional feedback is able to form the core of a robust oscillator. While delayed negative feedback is the foundation of the network, additional layers of autoregulation are required to refine the system's behavior. However, an important lesson from the analysis of our network design was that the basic oscillatory mechanism is driven by the negative feedback loop with a time delay caused by a cascade of cellular processes. A similar delay is observed in the *Drosophila* circadian clock, however little is understood about how it arises or what purpose it serves. This is an example of a clock characteristic that may be critical to the network's behavior, and lessons from synthetic biology could inspire further research into the role this mechanism plays in a native setting.

Another lesson from modeling our synthetic system relates to the degradation of the repressor. As expected, a critical requirement for sustained oscillations is the controlled decay of the key network proteins. This is observed to be true in native networks as well, as mutations that affect repressor degradation have a significant impact on the circadian period. However, an interesting result from the pairing of computational modeling and experimental data was the discovery of the importance of the enzymatic decay of the repressor. In order to accurately represent the dynamic behavior of the system, we found that the limited availability of the degradation machinery resulted in a first order, linear decay of oscillator proteins. This property of the network was found to be key in generating sustained oscillations. This type of discovery may lead to insight into the detailed workings of biological clocks. While it is well accepted that tightly controlled decay is important, the details about how this is regulated in native networks remains a mystery. This type of mechanism would be difficult to detect when buried in the complexity of an intricate biological network. Future studies focusing on the construction of synthetic circuits that isolate the cellular degradation machinery [60] may lead to further insights into the role of protease-mediated degradation [61,62] in regulatory motifs.

The success in building a network from the ground up that is capable of complex dynamic behavior demonstrates the power of engineering approaches for predicting and understanding biological behavior. Using these tools, we can mimic native network architecture and highlight key features that, while buried deep in the intricate regulatory web, are actually the driving force for fundamental cellular function. Future studies can

build upon this foundation, taking lessons from nature by incrementally increasing network complexity, adding layers of regulation, and monitoring the effects they have on the relevant characteristics of the system.

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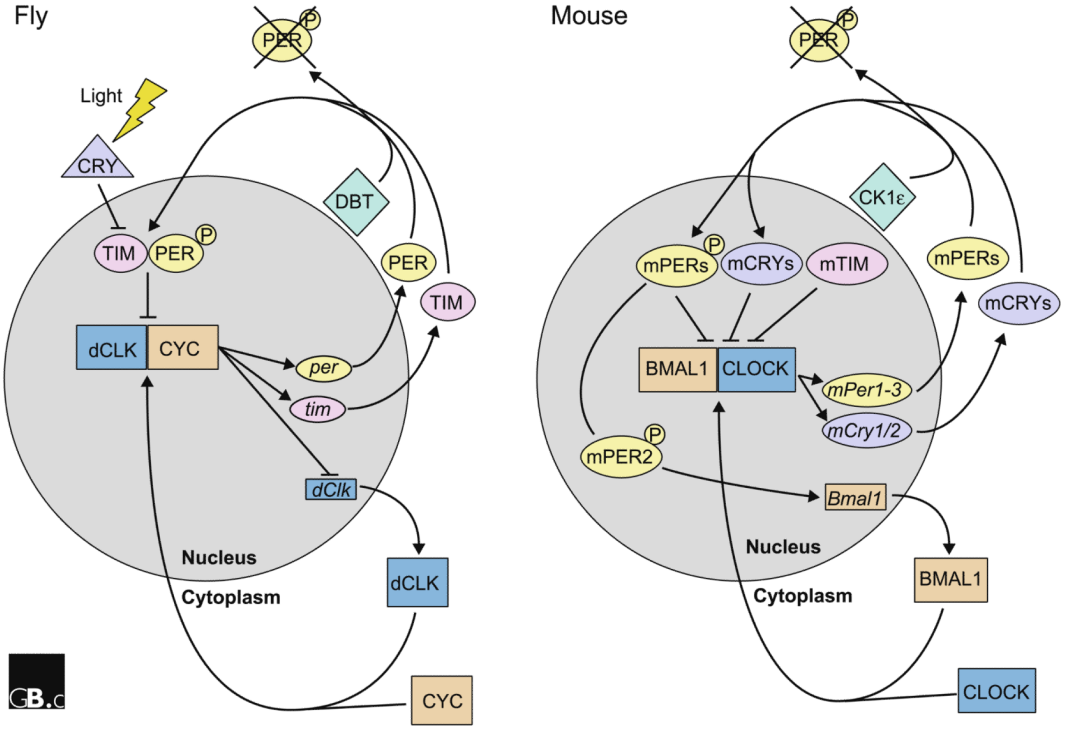

Fig. 1.

(From Hardin Genome Biology 2000 1:reviews1023.1 doi:10.1186/gb-2000-1-4-reviews1023). Model of the circadian clock circuits in *Drosophila* and mouse [29]. In *Drosophila*, CLK–CYC heterodimers bind to corresponding promoters and activate transcription of *per* and *tim* genes. PER protein is subsequently phosphorylated by DNT and CK2, which marks them for degradation. TIM binds phosphorylated PER and stabilizes it. The TIM/PER/DBT complexes are phosphorylated with the help of SGG kinase and bind to CLK/CYC, thereby removing them from *per/tim* promoters and thus repressing PER and TIM transcription. TIM/PER heterodimers, in turn, bind to the promoter of *clk* gene and upregulate its transcription.

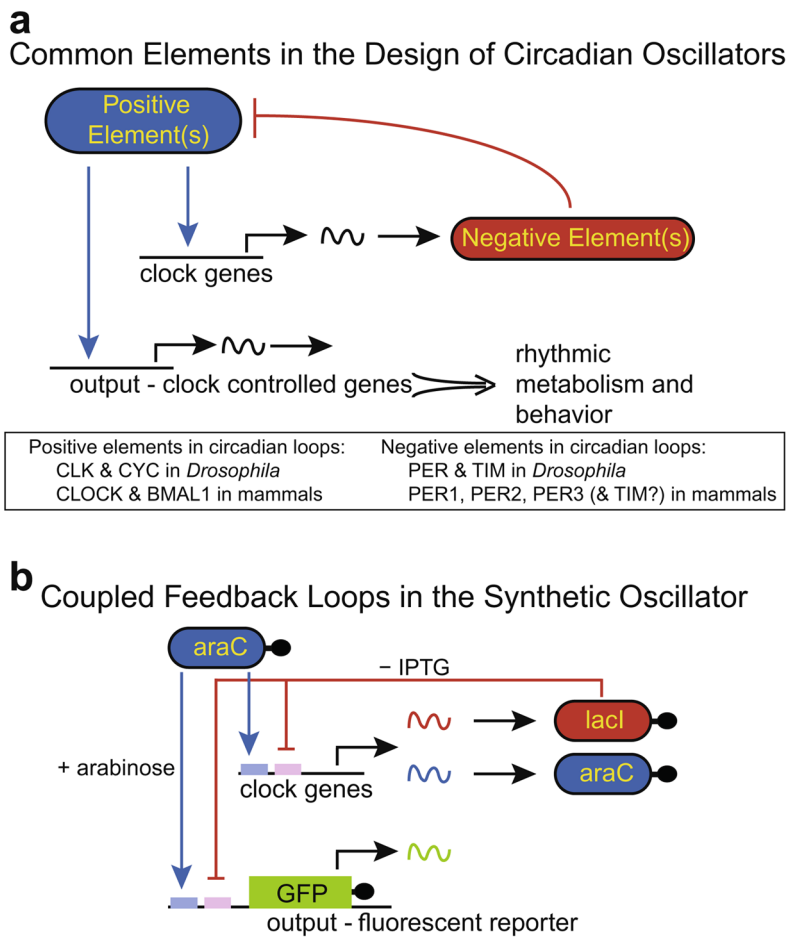

Fig. 2.

Design of circadian oscillators and a synthetic oscillator [56]. (a) (From Dunlap, Cell, Vol. 96, 271290, January 22, 1999) Necessary elements for a biological oscillator include negative and positive feedback loops. Proteins have been identified that serve these purposes in several well-characterized circadian oscillators [57]. (b) Network diagram of the dual-feedback synthetic oscillator. A hybrid promoter $P_{lac/ara-1}$ drives transcription of *araC* and *lacI*, forming positive and negative feedback loops.

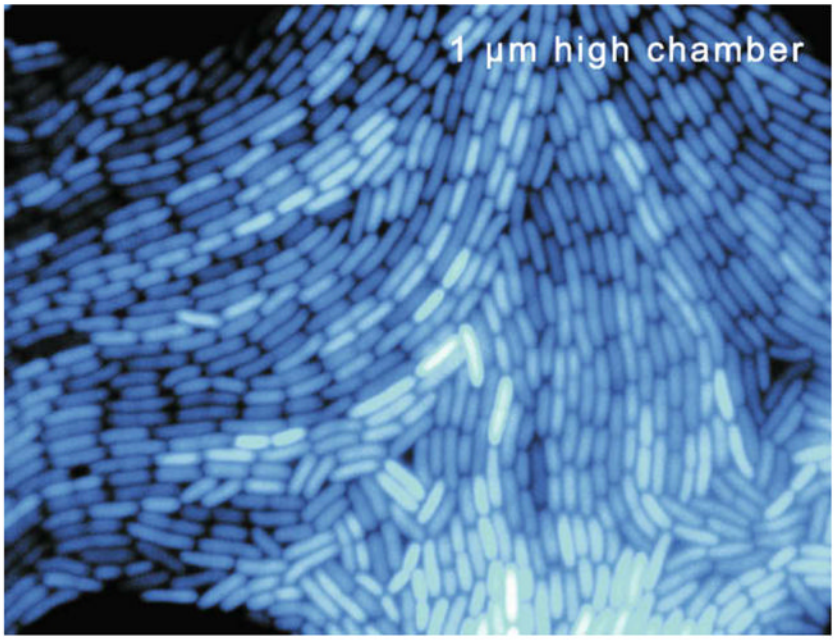

Fig. 3.

Microfluidic device tailored for single-cell imaging of a large population of *E. coli*. Shallow trapping regions confine cells to a monolayer. The device can be customized for different cell types: a 1 μm high *E. coli* trapping microchemostat is shown full of cells after 12 h of growth. Empty ovals in the trapping region are posts required to support the low height.

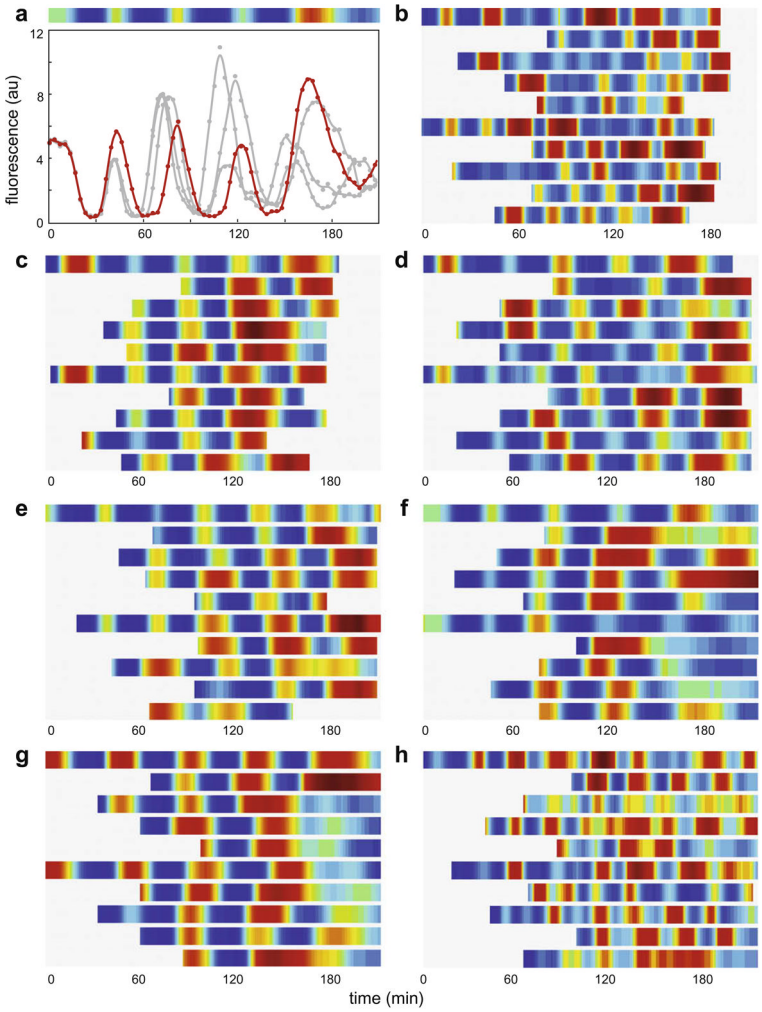

Fig. 4.

Oscillations in the dual-feedback activated repression circuit and the negative-feedback circuit [56]. (a) Single-cell fluorescence trajectories for the dual-feedback circuit induced with 0.7% arabinose and 2 mM IPTG. Points represent experimental fluorescence values, and solid curves are smoothed by a Savitsky–Golay filter. The trajectory in red corresponds to the density map above. (b–g) Single-cell density trajectories for the dual-feedback circuit for various IPTG conditions ((b) 0 mM IPTG; (c) 0.25 mM; (d) 0.5 mM; (e) 1 mM; (f) 2 mM; (g) 5 mM). *X*-axes are in min. (h) Single-cell density trajectories for the negative feedback circuit. The period was largely unaffected by IPTG concentration.

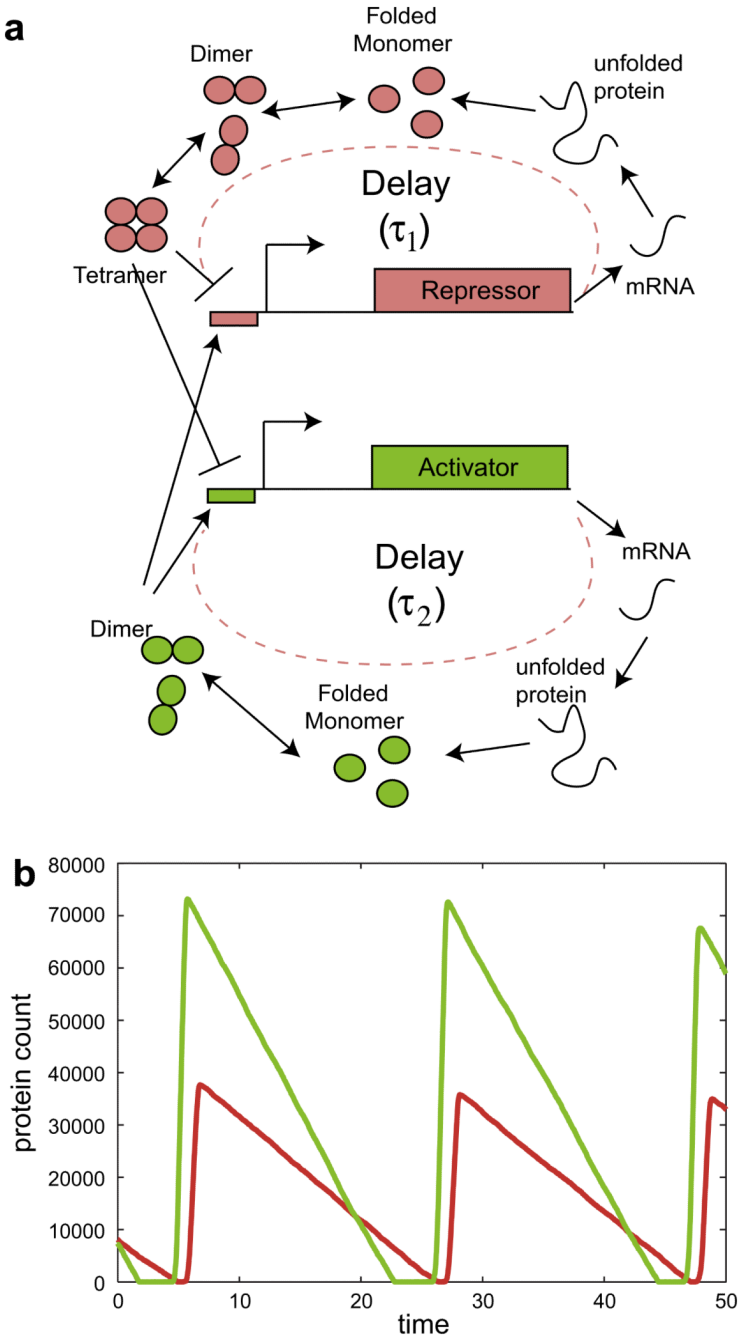

Fig. 5.

Degrade and fire oscillations in the dual delayed feedback model of activated repression [59]. (a) Network diagram of the dual delayed feedback oscillator. Sequential kinetic steps in forming functional transcription factors are replaced by single delayed reactions. (b) Time series of activator (green) and repressor (red).