Programming Microbes Using Pulse Width Modulation of Optical Signals

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Abstract

Cells transmit and receive information via signalling pathways. A number of studies have revealed that information is encoded in the temporal dynamics of these pathways and has highlighted how pathway architecture can influence the propagation of signals in time and space. The functional properties of pathway architecture can also be exploited by synthetic biologists to enable precise control of cellular physiology. Here, we characterised the response of a bacterial light-responsive, two-component system to oscillating signals of varying frequencies. We found that the system acted as a low-pass filter, able to respond to low-frequency oscillations and unable to respond to high-frequency oscillations. We then demonstrate that the low-pass filtering behavior can be exploited to enable precise control of gene expression using a strategy termed pulse width modulation (PWM). PWM is a common strategy used in electronics for information encoding that converts a series of digital input signals to an analog response. We further show how the PWM strategy extends the utility of bacterial optogenetic control, allowing the fine-tuning of expression levels, programming of temporal dynamics, and control of microbial physiology via manipulation of a metabolic enzyme.

Cells have evolved signal transduction pathways to fine-tune metabolic and physiological processes according to environmental conditions [1]. Many biological systems use temporal encoding of signals to propagate information, much like human engineered communication systems. Living organisms are composed of interconnected signalling networks that coordinate functions such as stress response, metabolism, cell cycle, and inflammation. These networks are composed of architectural motifs that have dynamic modulatory properties, including filtering of signals, oscillations, and hysteresis [2].

Given the importance of signal dynamics on biological function, precise control of gene expression is critical for living cells and for many applications in genetic engineering, where robust and predictive specification of protein levels is required to build and analyse complex systems [3]. Furthermore, strategies for easily programming signal perturbations allow for the mechanisms of how signal transduction pathway architectures respond to and modulate dynamically changing signals. From a bioengineering point of view, an ideal control strategy would allow rational, rapid tuning of expression over a range of levels and would allow for the kinetics of induction and repression of expression to be dictated experimentally. Optogenetic systems, in which gene expression is controlled by specific wavelengths of light via light-responsive receptors, are powerful tools for encoding spatial control of biological systems and have been used to manipulate neuron function, cell motility, and pattern formation [4–7].

Here, we sought to extend the usefulness and flexibility of a green-light-inducible system in *Escherichia coli* based on a two-component system from cyanobacteria (Fig. 1a) [8,9]. In the presence of green light, the histidine kinase ccaS phosphorylates the response regulator ccaR, increasing transcription

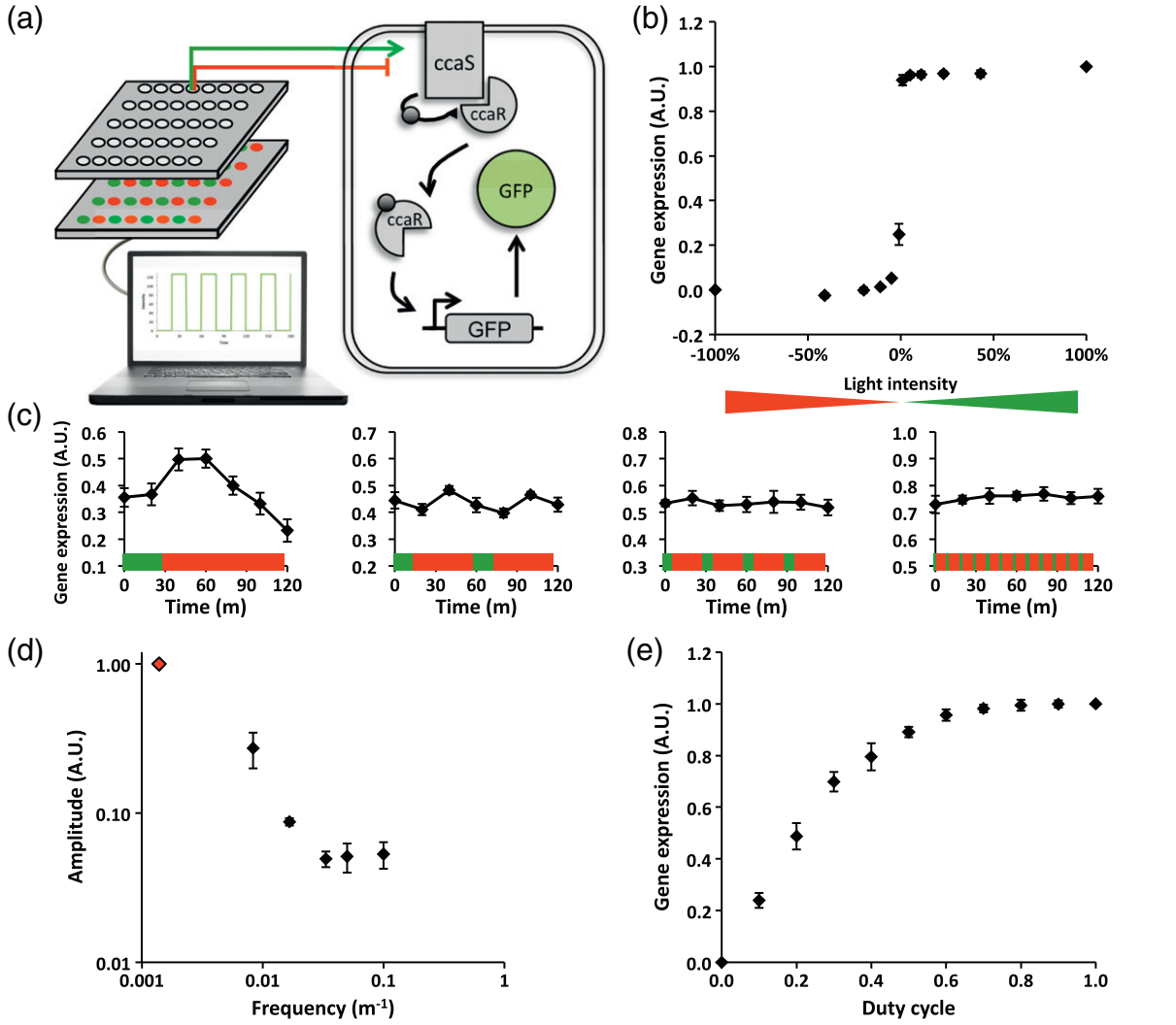

Fig. 1. PWM of optical signals allows graded control of gene expression. (a) An engineered hardware and genetic system for perturbing gene expression. A computer-controlled 8 × 12 array of dual (red and green) LEDs illuminates a 96-well, clear-bottom microplate. Red and green light from the LEDs is used to control the ccaS/ccaR two-component signal transduction pathway. Green light activates ccaS, increasing transcription of the GFP gene, while red light inhibits ccaS, decreasing transcription of the GFP gene. (b) The optogenetic system shows a sharp transition between repressed and induced states in response to input signal intensity. Light intensity is represented as a percentage of maximal LED intensity (approximately 30 W/m^2), with increasing red light intensity denoted by increasingly negative values for visualisation. (c) The GFP output of the system tracks input optical signal at low frequencies, but not at high frequencies. The pattern of applied light is represented within each graph in green and red. From left to right, cells were induced with oscillating green and red signals with a frequency equal to 0.0083 min^{-1}, 0.017 min^{-1}, 0.033 min^{-1}, and 0.1 min^{-1} and with a duty cycle of 0.25. (d) The relationship between input frequency and output amplitude indicates a low-pass filtering effect. The amplitude of oscillation is calculated as the difference between the maximal and minimal fluorescence values observed in (c), where 1.0 is the maximum difference between fully induced (green light) and fully repressed (red light) states. The frequency value for the 0.0014 min^{-1} data point (shown in red) is calculated based on a duty cycle of 0.25 and ~180 min required to transition from fully repressed to fully induced and represents the maximum amplitude. (e) System output shows a dependence on the duty cycle of oscillating input signals, allowing tuning along the entire range of expression. An oscillating square wave at a frequency of 0.1 min^{-1} was applied to cells. For all graphs, connecting lines are visual guides; gene expression points are an average of at least three independent GFP fluorescence measurements scaled from repressed (0.0) to induced (1.0) to show progression from "off" to "on" states; error bars are standard deviations.

of the reporter gene by approximately 2-fold (Supplementary Fig. 1). Red light inhibits this process, decreasing transcription. We developed an optical microtiter plate consisting of light emitting diodes (LEDs) corresponding to the induction and repression wavelengths of the cyanobacterial system (design

and construction detailed in Supplementary Information and Supplementary Fig. 2). The optical microtiter plate is computer controlled, allowing automated manipulation of optical signals over time. Each well can be programmed independently to enable a high-throughput workflow.

We first characterised the signal response of the optogenetic system by varying red and green light intensity in each well. The system shows repression under red light and induction under green light, with an extremely sharp transition between the two states (Fig. 1b). This is not optimal as a control strategy, as intermediate levels of gene expression are difficult to access by manipulating signal intensity.

Transforming a sharp response to a graded response is conceptually analogous to the conversion between digital and analog information in electronics. A common strategy to perform digital-to-analog conversion in control of electronic devices is pulse width modulation (PWM) [10,11]. PWM controls the power to a device by switching the supplied voltage on and off at rapid rates. The duty cycle (the fraction of time the voltage is on) determines the average power of the system. Given that duty cycle can be controlled digitally, PWM provides precise, linear control of the time-averaged power (an analog output). While PWM is widely used in electronics, it has not been used to control biological systems. Here, duty cycle represents the fraction of a wave period in which the LED is shining green light at maximum intensity, with the remainder of the period at maximum intensity red. The implementation of a PWM control strategy requires that switching between on and off signals occur more rapidly than the system can respond so that the system "averages" the pulse signal input to return an output. To examine the suitability of the optogenetic system for PWM control, we characterised the response to periodic input signals at varying frequencies (Fig. 1c). At low frequencies (less than 0.03 min^{-1}), the system tracked the input with a delay, resulting in an oscillation of output expression. At high frequencies, the system is unable to track the input signal. The magnitude of the oscillation amplitude is plotted against the signal frequency in Fig. 1d. The signal transduction system acts as a low-pass filter that attenuates input signals when the frequency is high and "passes" the input when the frequency is low.

At high input signal frequencies, the system cannot respond fast enough to track input signals for induction and repression, which is ideal for PWM control. We next examined the response of the system to varying duty cycle at high frequency (frequency = 0.1 min^{-1}). Reporter gene expression shows a graded response to duty cycle variation, allowing access to expression levels between the minimum and maximum states (Fig. 1e). This is a graded response at the single cell level, which is evident from flow cytometer assays of reporter gene expression (Supplementary Fig. 3).

The dynamics of gene expression in response to environmental and cellular stimuli are known to play an important role in biological regulation and adaptation [12]. In the mammalian NF-κB signalling pathway,

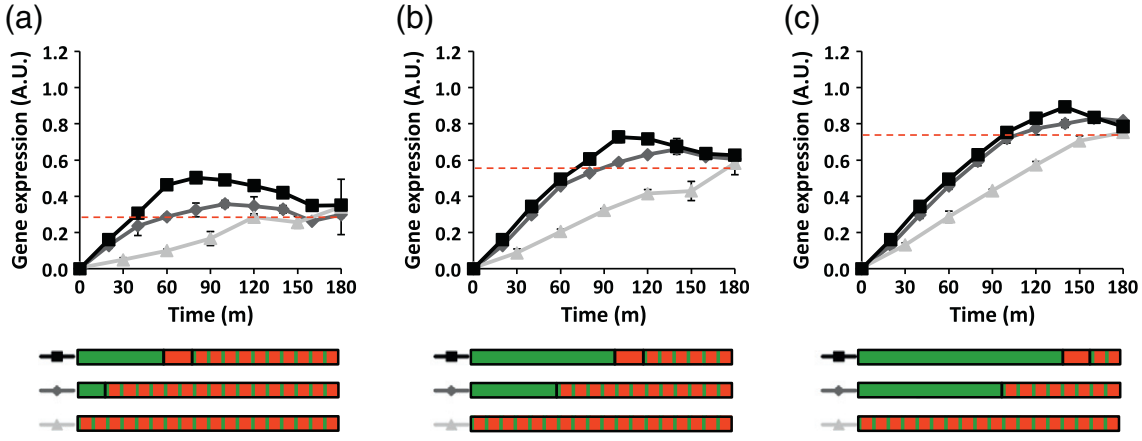

Fig. 2. Programming temporal dynamics of gene expression. Gene expression was programmed for slow (triangle), moderate (diamond), or rapid with overshoot (square) induction profiles, each targeted to three different final expression levels [low expression (a), medium expression (b), and high expression (c)]. Graph legends (below) show the induction/repression pattern for each gene expression curve. Green-filled blocks denote green-light induction; red-filled blocks denote red-light repression; alternating green/red are duty cycle induction [where duty cycle equals 0.1 for (a), 0.2 for (b), and 0.3 for (c), all at a frequency of 0.1 min^{-1}]. For all graphs, connecting lines are visual guides; red broken lines are visual guides indicating the target gene expression level; gene expression points are an average of at least three independent GFP fluorescence measurements scaled from repressed (0.0) to induced (1.0) to show progression from "off" to "on" states; error bars are standard deviations.

for example, different inflammatory stimuli induce distinct patterns of NF-κB activity [13,14]. The properties of these patterns include the rate of increase and decrease of expression, the "overshoot" kinetics, and the delay of induction or repression after a stimulus is applied. Experimental tuning of these properties is difficult to achieve with conventional inducible promoters, which has limited the understanding of how signals are propagated and "interpreted" by cellular networks. For example, it would be useful to characterise the biological effects of inducing gene expression at different initial rates that reach the same steady-state value. This would allow experimental tests of the relative contribution of the parameters of temporal dynamics on cellular signalling, metabolism, and physiology. Dynamic input–output studies of biological systems are valuable for revealing genetic network structure, parameters, and robustness [15–17].

We next sought to manipulate the dynamics of gene expression and to independently program temporal properties using a series of tailored pulsed signals. We tested whether the time to reach a target steady-state value could be tuned. Using experimentally derived parameters for the duty cycle transfer function (Fig. 1e) and the temporal induction and repression curves (Supplementary Fig. 4), we developed simple protocols programming gene expression to progress towards a pre-set steady-state value by three distinct induction profiles: slow, moderate, and rapid with overshoot. The program for a slow approach transitions directly from a repressed state (duty cycle = 0.0) to the final duty cycle. The program for a moderate approach applies maximal induction (duty cycle = 1.0) until 20 min prior to the time at which the desired final gene expression level is reached under maximal induction. The induction is then changed to the final state duty cycle to hold gene expression at the desired value. A rapid approach with overshoot is programmed using maximal induction (duty cycle = 1.0) until 20 min past the point at which the desired gene expression level was reached. At this point, transcription is repressed (duty cycle = 0.0) for 20 min and then the final state duty cycle is applied, causing the final gene expression level to be approached from above. These protocols were applied successfully to three different final gene expression levels (corresponding to duty cycles of 0.1, 0.2, and 0.3; Fig. 2a–c), demonstrating that a range of expression values and temporal dynamics can be accessed using this control scheme. In addition, a wide range of signal frequencies can be used to control gene expression using PWM (Supplementary Fig. 5).

The ability to precisely manipulate gene expression levels and dynamics is a powerful tool to perturb cellular metabolism and physiology. To further test PWM as a control methodology, we constructed a genetic system to place an *E. coli* metabolic pathway under the control of the optogenetic system. Light is an ideal tool to manipulate bacterial physiology, as it can be reversibly applied to a culture without physically perturbing the cells. We cloned the *metE* gene into the optogenetic system (Fig. 3a). *metE* is a methyltransferase involved in methionine biosynthesis and is an essential gene for growth in media lacking methionine. Thus, we predict that the growth rate of bacteria in culture media lacking methionine will be dependent on *metE* expression level. We introduced this construct

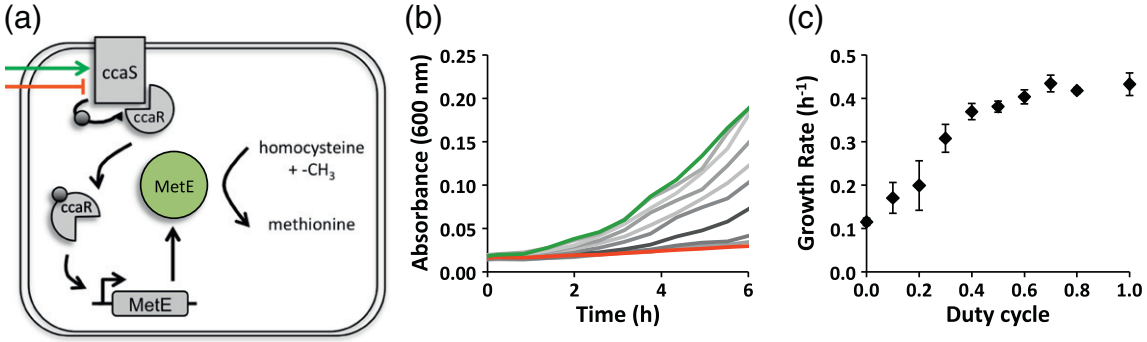

Fig. 3. Control of bacterial growth rate by PWM of a metabolic protein. (a) The *E. coli* methyltransferase *metE* was placed under control of the optogenetic system and transformed into a *metE* genomic deletion strain that does not grow in the absence of methionine (Supplementary Fig. 6). (b) Growth curves of the optically controlled *metE* strain in media lacking methionine, subjected to varying duty cycles of light perturbation. The red line indicates full repression of the system while the green line indicates full induction. Grey lines indicate cultures subjected to varying duty cycles, from 0.1 to 0.8. All lines represent absorbance averages for three independent cultures. (c) Growth rate is dependent on duty cycle applied, showing a graded increase in rate with higher duty cycle. Growth rate is calculated as described in the methods section. Growth rate data are averages from three independent growth rate calculations; error bars are standard deviations.

into a strain with a genomic *metE* deletion and controlled expression by varying the duty cycle of green and red light pulses. Engineered strains showed growth rates proportional to duty cycle applied (Fig. 3b and c), demonstrating that the PWM strategy can be used to control bacterial metabolism. A control strain expressing GFP (*g*reen *f*luorescent *p*rotein) rather than *metE* showed no growth in the absence of methionine, while the wild-type strain showed no growth response to light (Supplementary Fig. 6). The growth rates of engineered cultures at high duty cycles are similar to that of the wild-type strain, indicating that a wide range of growth rates can be achieved (Supplementary Fig. 6). We also note that the transfer function of the dependence of growth rate on duty cycle closely matches the GFP–duty cycle curve (Fig. 1e), indicating a reliable dose–response curve for both the fluorescent reporter and a metabolic output.

The interface between electronic and biological systems is a promising area with the potential to develop tools for probing living cells and to deliver novel applications. We have demonstrated that a control strategy from electronics, along with genetic and hardware engineering, is a viable methodology for programming gene expression levels, temporal dynamics, and cell physiology. We anticipate that PWM can be used in analogous biological systems, both bacterial and eukaryotic, where signal input can be rapidly switched relative to the system response and output, which likely encompasses a large class of electronic–biological interfaces. For example, the high-osmolarity sensing pathway in *Saccharomyces cerevisiae* is known to function as a low-pass filter and therefore should be amenable to analog control through PWM [18]. Given the transformative impact of digital information processing in electronics and communications, strategies to control biology using similar principles could enable a step change in synthetic biology.

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Conflict of Interest: The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2013.07.036.

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PWM, pulse width modulation; LED, light emitting diode.

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