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Using Two-Component Systems and other Bacterial Regulatory Factors for the Fabrication of Synthetic Genetic Devices

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Abstract

Synthetic biology is an emerging field in which the procedures and methods of engineering are extended to living organisms, with the long-term goal of producing novel cell types that aid human society. For example, engineered cell types may sense a particular environment and express gene products that serve as an indicator of that environment, or effect a change in that environment. While we are still some way from producing cells with significant practical applications, the immediate goals of synthetic biology are to develop a quantitative understanding of genetic circuitry and its interactions with the environment and to develop modular genetic circuitry derived from standard, interoperable, parts, that can be introduced into cells and results in some desired input/output function. Using an engineering approach, the input/output function of each modular element is characterized independently, providing a toolkit of elements that can be linked in different ways to provide various circuit topologies. The principle of modularity, yet largely unproven for biological systems, suggests that modules will function appropriately based on their design characteristics when combined into larger synthetic genetic devices. This modularity concept is similar to that used to develop large computer programs, where independent software modules can be independently developed and later combined into the final program.

Here, we will start by pointing out the potential usefulness of two-component signal transduction systems for synthetic biology applications, and will describe our use of the *E. coli* NRI-NRII (NtrC-NtrB) two-component system for the construction of a synthetic genetic oscillator and toggle switch for *E. coli*. Procedures for conducting measurements of oscillatory behavior and toggle switch behavior of these synthetic genetic devices will be described. Next, we present a brief overview of device fabrication strategy and tactics, and will present a useful vector system for the construction of synthetic genetic modules and positioning these modules onto the bacterial chromosome in defined locations.

Using two-component signal transduction systems in synthetic biology approaches

Two-component signalling systems have been studied intensively for about 20 years, and provide numerous examples of systems where the cellular physiology of the regulatory phenomena and the activities of the signal transduction components are reasonably well understood (Hoch and Silhavy, 1995). Certain aspects of these signal transduction systems make them particularly useful for synthetic biology purposes. Foremost among these is that many two-component systems are not essential for viability under most growth conditions, and instead control fairly small numbers of genes, that are only required under some stress condition that need not be applied. Thus, “closed” systems can be envisioned, where all of the natural stress response genes and regulated promoters are deleted and the two-component system functions solely in the synthetic genetic device.

The mechanisms of two-component signal transduction are also useful from a synthetic biology perspective. These systems contain a transcriptional activator whose activity is controlled by reversible phosphorylation. This feature allows the activation activity to be “tuned” by experimental manipulation of the transmitter protein activities leading to receiver phosphorylation and dephosphorylation. This can be obtained, for example, by manipulating growth conditions, by using mutant forms of the transmitter protein, or by using mutant forms of the receiver protein (transcription factor) that have different levels of constitutive activity. A variety of transmitter and receiver protein mutants with fixed output activities are available for some two-component regulatory systems, and for the others it should be possible to introduce mutations based on available data from these characterized systems.

The sensory and signal-transduction properties of certain transmitter proteins, in particular EnvZ, are distinct functions, allowing facile re-engineering for development of new sensors. It has long been known that the transmitter module of EnvZ, when fused to the transmembrane portions of cellular chemotaxis receptors, allows OmpR-dependant gene transcription in response to the ligands of the chemotaxis receptors (Jin and Inouye, 1993). The generality of this phenomenon has been extended by linking the EnvZ transmitter module to a light-sensing receptor (Levskava et al, 2005), and may be extended further by using altered chemosensory apparatus to widen the range of environmental stimuli that can control expression from OmpR-dependent promoters. Presumably, all of the many transmitter proteins that are structurally related to EnvZ could be re-engineered for altered sensory activity using the same approaches, allowing coupling of a variety of stimuli to various receiver proteins.

The NRI/NRII (NtrC/NtrB) two-component system controlling nitrogen assimilation in many bacteria has a number of particularly appealing features. First, the transcriptional activator, NRI~P, acts from an enhancer that is relatively position independent (Ninfa et al, 1987). The main positioning limitation is that the enhancer must be a minimal distance of about 70 base pairs from the site of polymerase binding (promoter). This position independence of the enhancer is very useful in that it simplifies the construction of promoters that are combinatorially regulated by different transcription factors. Furthermore, the concentration of activator required to drive gene expression depends largely on the strength of the enhancer sequences, allowing experimental manipulation of promoter activation by using natural strong or weak enhancers, or by mutation of a natural enhancer (Feng et al, 1995). Thus, promoters in a synthetic genetic device can be activated sequentially, just as the natural Ntr promoters are sequentially activated by increasing NRI~P during the cellular response to nitrogen limitation (Atkinson et al, 2002). A second very useful property of NRI~P is that the activating species is either a hexamer or heptamer of NRI subunits, thus providing a very high kinetic order of transcriptional activation (Lee et al, 2003). A third useful property is that certain NRI~P dependent promoters are completely silent in the absence of activator. Specifically, the *glnK* promoter of *E. coli* exhibits essentially no basal expression in the absence of NRI~P, whereas it is a very strong promoter in the presence of a high concentration of NRI~P (Atkinson et al, 2002). Certain other Ntr promoters, such as the *glnA* promoter have a powerful and tight Ntr promoter (*glnAp2*) coupled with a weak non-Ntr promoter (*glnAp1*) that provides a basal level of expression in the absence of NRI~P, and is repressed by NRI~P (Reitzer and Magasanik, 1985). Thus, one has the choice of very tight or somewhat leaky transcription, and there is no reason to suspect that this property cannot be “tuned” by mutation to provide a desired ratio of basal/activated expression. A wide variety of mutant forms of the transmitter protein are available, that differ in their ability to bring about the phosphorylation and dephosphorylation of NRI and thus allow “tuning” of the phosphorylation state in the context of a synthetic genetic device (Atkinson and Ninfa, 1992; Atkinson and Ninfa, 1993, Pioszak and Ninfa, 2003). Finally, NRI-dependent promoters utilize RNA polymerase

containing the minor sigma factor σ^{54} , and are completely silent in the absence of this sigma factor, which is not essential for viability (Hunt and Magasanik, 1985). This opens the possibility for even more elaborate devices in the future, that use independent signalling pathways to control the presence of NRI~P and σ^{54} , resulting in AND gate function from an Ntr promoter.

Using the NRI-NRII system to build a synthetic genetic clock

The basic circuit topology for the synthetic genetic clock is shown in Fig 1. The clock consists of two modules: the activator module and the repressor module. The activator module (left) consists of a promoter that drives the expression of activator, and is itself activated by activator. The activator also drives the expression of the repressor module (right), which produces repressor. The repressor protein blocks the expression of the activator module. Modeling of this circuit indicated that it had the potential to produce a variety of oscillatory outputs, ranging from sinusoidal oscillators to relaxation oscillators, as well as non-oscillatory steady states, depending on parameters (Atkinson et al, 2003, and unpublished data). Interestingly, modeling also indicated that for each set of parameters where the intact device produced oscillations, the isolated activator module would function as a toggle switch that displayed strong hysteresis of repression (Atkinson et al, 2003). The basic design of this clock is related to an earlier hypothetical clock (Barkai and Leibler, 2000), except that in the clock of Barkai and Leibler, the repressor protein antagonises the activity of the activator, as opposed to repressing activator expression.

The implementation of the design is depicted in Fig 2 (Atkinson et al, 2003). The activator module (left) consists of the *E. coli glnA* promoter region, driving the expression of NRI. The natural *glnA* promoter contains a strong enhancer (light blue boxes) and three low affinity activator binding sites that function as a band-limiter or governor (grey boxes, [Atkinson et al, 2002]). The region also contains two promoters (depicted as bent arrows in Fig 2): the upstream *glnAp1* promoter is repressed by activator binding to the enhancer, and the downstream *glnAp2* utilizes polymerase containing σ^{54} and is activated by NRI~P. To connect this module to the repressor module, “perfect” *lac* operators (dark blue boxes [Atkinson et al, 2003]), were added at two positions: immediately downstream from the *glnAp2* transcriptional start site, imitating the position of the *lacO1* operator in the *lacZYA* operon, and immediately upstream from position -162. The intention of this design was to permit Lac Repressor to form a DNA loop and simultaneously contact both operators, as it does in repressing the *lacZYA* operon (Oehler et al, 1990). However, subsequent results have suggested that a repression DNA loop does not form, although the upstream operator is still required for good oscillatory function (unpublished data). The repressor module consists of the *glnK* promoter region and mRNA leader sequence, fused to the structural gene for Lac repressor, *lacI*. To facilitate the fusion, the initiation codon for *lacI* was converted to AUG. The *glnK* promoter has essentially no basal expression in the absence of activator, and requires a high concentration of activator for expression owing to its weak enhancer. The use of the Lac Repressor as the clock repressor permits easy synchronization of the cells with the lactose analogue IPTG. To bring about phosphorylation of NRI, the strain containing activator and repressor modules was transformed with the pBR322-derived p3Y15, which contains the mutant *glnL2302* allele for the transmitter protein NRII, driven from its natural promoter. The product of the *glnL2302* allele, NRII2302, is defective in bringing about the dephosphorylation of NRI~P, but is fully functional in autophosphorylation and phosphotransfer to NRI, ensuring the NRI is highly phosphorylated regardless of environmental conditions (Pioskasz and Ninfa, 2003).

The expected function of the circuit is as follows: When repressed, neither activator nor repressor are synthesized, and their levels will decrease by dilution as cells grow and divide.

Eventually, the concentration of repressor will decrease below the threshold for repression of the *glnA* control region, and the *glnAp1* promoter will drive the expression of NRI. This NRI will be quickly phosphorylated, and will repress the *glnAp1* promoter and activate the *glnAp2* promoter, leading to a dramatic increase in the concentration of NRI~P. Eventually, the activator will reach a high enough concentration to activate the *glnK* promoter, leading to a burst of repressor synthesis. This will continue until the concentration of repressor is sufficient to repress expression of the activator module, and eventually the system will be returned to its repressed state. To monitor oscillations, the repression of the *lacZYA* operon and the activation of Ntr genes, such as the natural *glnA* gene can be monitored by measuring the enzymes β -galactosidase and glutamine synthetase as the clock-containing strain grows in continuous culture (12).

Fabrication of the synthetic genetic clock

A unique aspect of our synthetic genetic clock is that the activator and repressor modules are not contained on plasmids in the cell, but rather are integrated into defined chromosomal locations, referred to as “landing pads” (Atkinson et al, 2003). We expect that this should provide a stable copy number to the modules, and indeed allows subtle manipulation of the copy number by using different locations on the chromosome, as the copy number of genes in rapidly growing cells displays a gradient from the origin of replication to the terminus of replication. The landing pads were designed to have nearby selectable markers that facilitate transfer of the modules between strains using standard P1 vir-mediated generalized transduction. The basic features of landing pads include bracketing the synthetic genetic module with transcriptional terminators, to prevent transcription from outside promoters. Two landing pads, located in the *rbs* and *glnK* regions of the chromosome, were used to integrate the activator and repressor modules into the chromosome. These landing pads were described previously and are available in the plasmids pRBS3 and pDK11, respectively (Table 1). Some of the features of these landing pads are provided in Table 1. Since the fabrication of our synthetic genetic clock, we have devised a convenient plasmid system for construction of new landing pads and for modular construction of synthetic genetic devices in these landing pads. This vector system will be described in a later section. Our original fabrication methods for building the synthetic genetic clock modules consisted of standard molecular biology methods including PCR, site-specific mutagenesis, etc. and were described previously (Atkinson et al, 2003). In a later section, we will describe improved methods and materials that should ease the construction of synthetic genetic devices.

Assembly of the clock strain was as follows (strains listed in Table 1): The starting strain 3.300 contains a *lacI* null mutation. The *glnA::Tn5* mutation was then introduced by P1 vir-mediated generalized transduction, with selection for kanamycin resistance and screening for glutamine auxotrophy, forming strain 3.300A. A deletion of *glnL* and *glnG* was then introduced by transducing 3.300A with phage grown on strain SN24 (*glnLglnG*), with selection for glutamine prototrophy (*glnA*⁺) and screening for kanamycin sensitivity, producing strain 3.300LG. Strain 3.300LG is thus *lacI glnL glnG* but *glnA*⁺ and *lacZYA*⁺, and serves as the host for the synthetic genetic clock.

To recombine the clock genetic modules onto their chromosomal landing pads, a *recD* mutant is transformed with linearized plasmid DNA, where the site of cleavage is within the plasmid vector sequences. We have had success using strain TE2680 (Elliott, 1992), and have observed that the *recD::TN10* mutation (conferring tetracycline resistance) can be transduced from strain TE2680 into a variety of strain backgrounds, permitting transformation with linear DNA. We typically use electroporation of the linearized DNA to obtain a large number of transformants, and use up to 1 μ g of DNA per transfection. The drug-resistance marker of the landing pad is selected (Table 1), and the transformants are

screened for resistance to ampicillin, which is encoded by the plasmid vector sequences. Transfectants that have the landing pad drug resistance but lack resistance to ampicillin are those in which the landing pad has recombined onto the chromosome. Transducing phage are then grown on these recombinants, and the integrated landing pad is introduced into desired strains by selection for the associated drug resistance of the landing pad. Thus, to build the genetic clock, strain 3.300 LG was sequentially transduced with *rhs:glnApOG6..gent* (introducing the activator module) and *glnK:pglnKlacI2...cam* (the repressor module), with selection for gentamycin resistance and chloramphenicol resistance, respectively. This strain, referred to NC12 was then made competent by standard procedures and transformed with p3Y15 (encoding NR2302), with selection for ampicillin resistance, forming strain NC12/p3Y15, which is then used for clock studies. We have observed that the final plasmid-containing strain can be stored as a freezer culture at -80°C , and reproducible clock experiments can be performed by streaking a few ice crystals containing the frozen cells on LB medium containing ampicillin immediately prior to the experiment, and picking a single colony isolate for the experiment. This strain and the intermediate strains and modules (Table 1) can be obtained from the authors.

Functions of the individual clock modules

The activator module and repressor module functions can be independently measured in intact cells. The activator module, when present in cells containing wild-type NR2 and wild-type *lacI* encoding Lac Repressor, is predicted to form an N-IMPLIES logic gate with respect to ammonia and IPTG (Fig 3). In the presence of wild-type NR2, the nitrogen-rich state brought about by the presence of ammonia causes the formation of the NR2-PII complex and the rapid dephosphorylation of NR2-P (18). Furthermore, in the absence of IPTG, the constitutively-present Lac Repressor blocks the expression of the activator module. Expression of a reporter consisting of the *glnK* promoter to *lacZ*, or any other Ntr gene, thus requires the presence of IPTG and the absence of ammonia, providing the N-IMPLIES logic function. After construction of the bacterial strain, we observed that the cells indeed only expressed β -galactosidase in the absence of ammonia and the presence of IPTG.

The activator module also displayed toggle-switch function in the absence of the repressor module, as predicted by modeling (Atkinson et al, 2003, Fig 4). For these experiments, where only repression of the activator module was examined, the activator module was integrated into a strain deleted for both *glnL* and *glnG*, and the transmitter protein function was complemented by the plasmid p3Y15, encoding NR2302. The strain also contained a wild-type *lacI* and *lacZ*, but contained a mutation of *lacY*. The mutation of *lacY* is essential to allow control of the internal IPTG concentration by variation in the external IPTG concentration (Novice and Weiner, 1957). The assembly of the strain was as follows: strain M7044 (*lacY*) was transduced to *glnA::Tn5* by selection for kanamycin resistance and checking for glutamine auxotrophy, forming strain M7044A. This was then transduced to glutamine prototrophy using phage grown on strain SN24 (*glnLglnG*), producing strain M7044LG. This strain was then transduced with the activator module, by selection for gentamycin resistance of the integrated *rhs* landing pad, producing strain TS1. Finally, strain TS1 was made competent by standard methods and transformed with plasmid p3Y15, with selection for ampicillin resistance.

Toggle switch experiments are performed by growing the strain overnight in the absence and presence of 0.1 mM IPTG, resulting in naive and induced cultures. The overnight cultures are then diluted one million-fold into media containing various concentrations of IPTG. Growth is continued for about 15–17 generations, until the cultures were in mid-log phase, and β -galactosidase and glutamine synthetase are measured. The glutamine synthetase measurement provides an indication of the level of activator, while the β -galactosidase

measurement provides an indication of the level of repressor. It is expected that the naive and induced cultures should show equivalent levels of β -galactosidase expression, but, if there is hysteresis in the activator module control, should show very different glutamine synthetase levels. As shown in Fig 4, this behavior was observed. It should be noted that since the *glnL2302* mutation was present on p3Y15, a variety of growth media can be used for the experiment, including complex media such as LB or nutrient broth. To compensate for the loss of normal Ntr regulation, glutamine is included in all media at 0.2% (w/v). [Note that glutamine does not survive autoclaving and filter sterilized glutamine must be added to the medium after autoclaving.]

The repressor module, when present in cells containing a deletion of the natural *lacI* and wild-type *lacZYA* and Ntr system, is predicted to display OR gate function with regard to ammonia and IPTG control of *lacZYA* expression (Fig 5). This is because ammonia blocks the formation of the activator, while IPTG blocks the function of Lac Repressor produced from the repressor module. Consequently, either stimulus provides full expression of *lacZYA* (Fig 5). The designed strain (3.300 containing the repressor module) was observed to indeed show the OR gate function.

Procedures for clock experiments

We have used a variety of growth media for clock experiments; a very good medium providing rapid growth of the cells and good oscillatory function is W-salts based glucose-glutamine-caseamino acids, with the following formula (per liter): 10.5 g K_2HPO_4 , 4.5 g KH_2PO_4 , 0.65 mL of 1 M $MgSO_4$, 0.04 g thiamine, 0.04 g tryptophan, 0.5 g glutamine, 10 g glucose 5 g Bacto caseamino acids, 0.1 g ampicillin, and 1 mL of 34 mg/mL chloramphenicol. The medium is filter sterilized, owing to its labile components. A single colony of the clock strain is picked from an LB + ampicillin plate and inoculated into 2 mL of medium, and allowed to grow to saturation. 1.2 mL of the overnight culture is used to inoculate 120 mL of medium containing 0.5 mM IPTG. This culture is incubated until the turbidity reaches the desired turbidity for the clock experiment; typically 10–12 hr incubation provides an OD_{600} of ~0.6, which gives good results. The cells are harvested by centrifugation, resuspended in 120 mL of fresh medium lacking IPTG, pelleted, again resuspended in 120 mL of fresh medium lacking IPTG, and introduced into the continuous culture device. The volumes stated above can be scaled as appropriate for a variety of working volumes required by different fermentors. The optical density of the culture can be corrected in the initial stages of the run by control of the fermentor nutrient pump. Best oscillatory behavior was observed when the continuous culture was run at OD_{600} of 0.5–0.6. Cells are grown in the continuous culture device at a constant optical density by controlling the nutrient flow. Samples are removed periodically (or obtained from the fermentor efflux) and assayed for β -galactosidase and glutamine synthetase.

We have used two methods for conducting the continuous culture experiments, one utilizing a standard laboratory fermentor that was not designed to function as a turbidostat, and the other using a custom-built turbidostat. Both methods will be briefly described here. It is important to note that the clock strain does not grow at a constant rate in continuous culture; rather, the presence of the synthetic genetic clock causes growth to slow down as the activator module and *lacZYA* expression are derepressed and to speed up as the activator module and *lacZYA* are repressed. Thus, to maintain a constant culture optical density, the nutrient flow must be continuously adjusted. When using a standard laboratory fermentor not designed to function as a turbidostat, this is accomplished by periodically sampling the fermenter efflux, measuring OD_{600} , and manually adjusting the nutrient flow rate so as to hold the culture at constant turbidity (12). Since clock experiments typically are run for 80 hrs or more, the experiments require lots of coffee and/or several people to take shifts minding the fermenter. Nevertheless, good oscillatory function can be easily observed in

such experiments, despite the fairly crude control of the culture turbidity (Atkinson et al, 2003, Fig 6).

For experiments with a typical laboratory fermentor, the starting culture at approximately the desired working OD₆₀₀ is introduced directly into the reactor, and the optical density is corrected to the desired optical density by manipulation of the nutrient pump. Filtered air is pumped into the reactor and vigorous stirring is used to provide good aeration. Medium is pumped into the reactor from a 4L reservoir, to which fresh sterile medium is added aseptically as needed. Samples are collected from the efflux line directly into a clean disposable cuvette; the OD₆₀₀ of the culture is measured, and aliquots are used for the β -galactosidase and glutamine synthetase assay (12).

To automate the clock experiments, we developed a turbidostat as depicted in Fig 7. The reactor is a 500 mL Erlenmeyer flask that contains a 120 mL culture that is kept at constant optical density by varying the media flow appropriately. A close-up view of the reactor is shown in Fig 8; it has four connections to the outside: one line to pour media in, one line to suck culture out when the volume slightly exceeds 120 ml (waste line), one line to take samples, and one line to pump in filtered air. The air pump is a large fish tank aerator connected to a standard 0.22 μ m filter to provide sterility. The reactor is contained within a standard small laboratory incubator, and good mixing of the culture is provided by placing the flask on a standard stir plate and including a magnetic stir bar in the vessel (not depicted). In addition to the stirring, the pumping in of air contributes to mixing within the reactor.

The media is pumped in from a media reservoir at a variable speed. To maintain sterility and accommodate the large volumes of medium required, we use a home-made medium “tree” that consists of up to 6 one-gallon bottles connected in a daisy chain by sterilizable tygon tubing. This apparatus is sterilized dry under a tin-foil tent, placed into a bacteriological hood while still hot, and the filter sterilized medium is introduced after it cools sufficiently. The waste line sucks culture out and has twice the flow rate of incoming media, and it sucks culture out only when the volume goes higher than 120 mL. The waste line needs to have a stronger flow rate than the media line in order to keep the volume constant. The depicted design was chosen, as opposed to having the same flow rate of media in and culture out, because fluctuations in the flow rate of the lines, even if both lines are connected to the same pump, makes the control of the volume more difficult. Any slight difference in flowing rates may lead to volume accumulation or reduction, and since each experiment lasts several days, this constitutes a problem. The waste flow rate is made double to incoming medium flow rate by using tubing of twice the diameter and the same pump (Fig 7).

The sampling pump (Pump 2 in Fig 7) intermittently removes culture from the fermentor at a rate that is considerably less than the nutrient flow. Every five minutes a small aliquot of culture is pumped out through the sampling line to a flow cell (Fig 7). The flow rate of media is changed as a function of this OD₆₀₀, in order to keep the optical density constant. After each OD₆₀₀ reading, the sampling pump is shut off, and the flow cell is washed with sterile water, using water that is pumped into the sampling line from a water reservoir by the wash pump (Fig 7). This wash routine was found to be important, as continuous pumping of the culture through the flow cell and autosampler leads to the formation of a biofilm on the surfaces, fouling the reading of absorbance and contaminating the samples and instruments. Every 30 min, or six OD₆₀₀ readings, a 2 mL sample of the culture is directed to waste to clear the lines and a 1 mL sample is collected in a well of a deep well 96-well microtitre plate. The automatic sampler allows the sampling line to point to any of the 96 wells in the microtitre plate, or to waste.

The system depicted in Fig 7 is fully computer-controlled. The media, sampling and wash are each pumped by a computer controlled peristaltic pump (media and sampling lines are pumped by VS-series Alitea peristaltic pumps, water is pumped by a S-series Alitea peristaltic pump), while the spectrometer (Ocean Optics SD2000) and autosampler (AIM 3200) also have an interface to the computer. The flow cell to measure absorbance, as well as the pumps, autosampler and spectrometer were purchased from FIAlab Instruments (Bellevue WA).

The software used to control the system was implemented in Labview (National Instruments, Austin TX). Labview is a graphical programming language that eases the interface of the computer with instruments and data acquisition devices. Some instruments, like the autosampler and valves, have an interface to the computer via the serial port and an ascii language, while others like the spectrometer or a data acquisition card, have some other way of interfacing with the computer, but they offer a basic library of functions in Labview to operate them. Essentially any instrument can be controlled from Labview. Besides its ease to interface with instruments, as a programming language Labview offers the capabilities of any other programming language (like C/C++ or Java). Using Labview, we designed the following algorithm to run the system (collect samples and keep the OD constant)

- Set the target OD and the media flow rate (measured as the percentage of the top speed in the pump, initially 50%)
- Every five minutes:
 - Pump culture from the flask to the flow cell, and make an OD measurement
 - Wash the flow cells with sterile water
 - Adjust the media flow rate according to the formula

$$new\ flow = flow \times \left(1 + \frac{OD - target\ OD}{target\ OD} \right)$$

- Every half an hour (every 6 readings), pump 2 mL of culture to waste and collect 1 ml of culture in a deep well 96-well plate.

Conducting automated experiments requires that the samples be maintained at low temperature (4 °C) to prevent growth of the cultures and changes in the level of β -galactosidase and glutamine synthetase. We observed that samples held at 4 °C displayed stable levels of these two enzymes for several days. To provide uniformity, we routinely maintain all samples at 4 °C for at least 4 hrs before conducting the assays. The autosampler is contained within a standard small refrigerator, which we adapted to our purpose by drilling a small entry port into one side and a small “overflow” port into its base. The entire apparatus, including refrigerator, incubator, computer, pumps, and flow cell easily fits onto one standard laboratory bench.

The automated system utilized miniaturized assays, and uses samples that have been stored at 4 °C. We observe that the miniaturized assays are somewhat noisier than the standard assays, and that some of this may be due to inconsistent resuspension of the settled cells from the stored samples. Thus, we recommend special attention to resuspending the settled cells, by both extensively vortexing the collection plates and pipetting the samples into and out of the well several times before taking the aliquots for measurement. This noisiness notwithstanding, oscillatory behavior can be easily observed using an automated system with samples stored at 4 °C and miniaturized assays (Fig 9).

Procedures for the measurement of glutamine synthetase and β -galactosidase for clock and toggle-switch experiments

Glutamine synthetase microassay

1. A reaction mix is assembled as follows:

Reagent	Amount (mL)
0.45 M Imidazole pH 7.33	35
0.3 M $\text{NH}_2\text{OH}\cdot\text{HCl}$	7
0.01 M MnCl_2	3.5
0.06 M KAs_2O_4 pH 7.2	35
6 mM ADP, pH 7.0	7
1.5 mg/ml CTAB	7
2.9 % glutamine	12
Correct the pH to 7.27 with KOH.	

2. Transfer 400 μL of each sample to be assayed to a well in a deep well 96-well plate. Centrifuge to pellet the cells and carefully remove the supernatant and discard. Resuspend the cells in 40 μL of CTAB cell-wash buffer, and transfer to a standard flat-bottom 96 well microtitre plate. The CTAB cell-wash buffer is: 5 mM Imidazole pH 7.15, 0.1 mg/mL CTAB, 0.27 mM MnCl_2 .
3. 100 μL aliquots of the reaction mix are pipetted into the wells; this starts the reaction. Typically, the mix aliquots are added at 20 sec intervals using an 8-channel micropipettor, and later the reactions will be stopped in the same sequence at 20 sec intervals. When all samples have been added, the plate is incubated at 37 °C in a standard microbiological incubator. In a separate plate, a sample from an early time point in the clock experiment (where the glutamine synthetase activity is expected to be high) are set up in triplicate in three separate wells of a microtitre plate. This plate will be used to determine the appropriate length of time to incubate the reactions. At 30 min intervals, stop solution is added to one of the three wells in the test plate, and the reactions are allowed to continue until a dark brown color is obtained upon stopping the test reaction. Typically, a one-hour incubation is sufficient for measurement of the activity. The stop solution is (per L): $\text{FeCl}_3\cdot 6\text{H}_2\text{O}$ 55 g; TCA 20 g, HCl 21 mL.
4. To stop the reactions, 100 μL of stop solution is added to each well, in the same sequence and time interval that the reactions were started. Upon addition of the stop solution, the reaction mixtures that contain high glutamine synthetase activity will turn dark brown. Since the cell samples should all have similar OD_{600} , the oscillations in GS level should become immediately apparent as the reactions are stopped.
5. The OD_{540} of the stopped reactions are directly measured in a plate reader. In a separate plate, the OD_{600} of the original cell samples are directly read. The glutamine synthetase activity, in arbitrary units, is simply the OD_{540} value divided by the OD_{600} value.
6. For toggle-switch experiments, the cells from 1 mL of the cultures are pelleted in microcentrifuge tubes, and resuspended in 0.1 mL of CTAB-cell wash buffer. Aliquots of 20 μL and 50 μL are then added to the reaction mix in microtitre plates, and the procedure described above is followed.

β -galactosidase microassay

1. The wells of a deep-well 96-well plate are loaded with 400 μ L of Z-buffer, 10 μ L of 0.1% SDS, and 10 μ L of chloroform (add last). The formula for Z-buffer is (per liter): 16.1 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 5.5 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.75 g KCl, 0.246 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Immediately prior to the assay, add 0.27 mL of β -mercaptoethanol/100 mL of Z-buffer.
2. A 100 μ L of the culture sample is added progressively to each well and mixed thoroughly by pipetting (the chloroform must be well mixed into the suspension for uniform cell permeabilization). We typically use an 8-sample multichannel micropipettor to minimize the difference in exposure of samples to the chloroform. The reaction mixtures are then incubated 10 min at room temperature.
3. To start the reactions, 100 μ L of ONPG (4 mg/mL in 0.1 mM phosphate buffer) is added to the wells in sequence and at fixed intervals, and mixed thoroughly by pipetting.
4. Allow the reaction to proceed until the appearance of yellow color in some of the wells. Note that oscillations in the level of β -galactosidase becomes readily apparent at this stage, and some of the wells should never turn bright yellow since they contain very low levels of β -galactosidase. Typically, a 5–10 min incubation is required.
5. Reactions are stopped in the sequence they were started and at the same intervals, by adding 200 μ L of 1M Na_2CO_3 .
6. The stopped reactions are allowed to sit for 10 min for full color development and settling of the cells, then 150 μ L aliquots (from the top) from each well is transferred to a 96 well flat bottom plate and the OD_{410} and OD_{560} are recorded. In a separate series of wells, the OD_{600} of the original culture samples is measured.
7. The β -galactosidase activity is $[\text{OD}_{410} - (1.75 \cdot \text{OD}_{560})]/(\text{OD}_{600} \cdot \text{time})$.

Improved procedures for the fabrication of synthetic genetic modules and integration of these modules into chromosomal landing pads

During our synthetic biology studies, we have developed fabrication methods in concert with development of the clock, such that early versions of the clock do not incorporate the most useful aspects of fabrication methodologies developed later. Here, we will present our most recent vector system for the fabrication of synthetic genetic modules and incorporation of the fabricated modules into chromosomal landing pads. The vector system should allow the placement of any module in any position and on either strand of the chromosome, and permit the genetic modules to be easily constructed from diverse natural components.

To develop new landing pads, the vectors shown in Fig 10 can be used. A non-essential and easily scored gene, such as a sugar utilization gene, is chosen as the target for chromosomal integration. Two fragments of the gene of ~90 bp each are cloned between the EcoRI and SacI sites, and between the XhoI and HindIII sites. The landing pad will be localized by recombination of these sequences with their chromosomal counterparts; we typically choose “right” and “left” target sequences from within the same gene. Next, we introduce an antibiotic resistance gene flanked by strong transcriptional terminators between the SacI and NotI sites, as depicted in Fig 11 (Step1 plasmids). We have used kanamycin resistance and chloramphenicol resistance in Step 1 plasmids; we refer to this marker as “AB1”. Step 1 plasmids are functional landing pads that can be used to integrate modules into the target gene, those available and under construction are listed in Table 1. One clones the genetic

module to be placed onto the chromosome between the remaining 5 unique sites that are flanked by the recombination target fragments. One could introduce the module as a unit after assembly in another plasmid, or sequentially add portions. Typically, we use the NdeI site for the beginning of the structural gene, since NdeI sites contain an ATG initiation codon. If the structural gene is cloned as an NdeI-SalI fragment or as an NdeI-BamHI fragment, a strong terminator can be added between the SalI and XhoI sites, isolating the gene from extraneous transcription from both directions. Next, the promoter and mRNA leader sequence are added as a NotI-NdeI fragment, completing the module. Typically, this fragment is assembled in separate plasmids, or added as a synthesized DNA fragment by annealing long oligonucleotides that have been designed to have the appropriate overhangs. We have worked with synthesized fragments of length 100 bp, which permits the construction of many combinatorial promoters/leader sequences. All of our vectors contain a unique PstI site in the vector sequence that allows easy linearization of the plasmid for electroporation into a *recD* mutant. In the event that there is a PstI site in the module itself, several other unique sites are also present in the vector portion of the molecule, that might be used.

Synthetic genetic devices can be built up by placing different modules into different chromosomal landing pads, as was done for our initial clock. The problem with this approach is that each module is linked to a distinct antibiotic resistance marker, and one quickly runs out of antibiotic resistance selections that offer very strong selection in the presence of each other. Tetracycline resistance provides a strong selective marker, but we exclude its use from our system since we use the *recD::Tn10* (tet resistant) mutant to recombine modules onto the chromosome. Similarly, our plasmid vectors encode ampicillin resistance, and thus we do not use this drug resistance marker elsewhere in the system. We have found that kanamycin resistance and chloramphenicol resistance provide very strong selection (Table 1), and that gentamycin resistance, as found in our pRBS landing pad (Table 1), does not. Thus, integrating three separate modules onto the chromosome is a problem. To overcome this, two approaches are taken. First, we are currently investigating additional antibiotic resistance markers, such as spectinomycin resistance, for use as “AB1” in Step 1 plasmids. Second, we designed the vector system such that each landing pad can be used in a reiterative fashion by sequential integration of modules, which end up next to each other on the bacterial chromosome. The plasmids used for these successive integrations, pStep2, pStep3, and pStep4 are shown in Fig 11. As before, there are two versions of each of these to permit either orientation of the genetic module. The target sequences of the pStep2-pStep4 plasmids consist of internal segments of the drug resistance genes used in the prior step. Thus, if one uses a pStep1 plasmid with kanamycin as the selectable marker (AB1 in Fig 11), the corresponding pStep2 plasmid will integrate into the AB1 gene and contain as a selectable marker chloramphenicol resistance (AB2). The pStep3 plasmid will contain AB1 as the selectable marker, and integrate into the AB2 gene, and the pStep4 plasmid will contain the AB2 gene as a selectable marker, and integrate into the AB1 gene. Thus, the drug resistance of the module is switched between AB1 and AB2 upon each successive integration, and the synthetic genetic constructs are each flanked by terminators and located next to one another on the chromosome. A simple PCR test of the final strain can then confirm the expected structure. The sequence of nesting is depicted in Fig 12 as well as the predicted end product of an assembly where 4 promoter-gene fusions (G1-G4) are positioned in a single landing pad.

Fabricating genetic modules

Most of the genetic modules that we fabricate consist of a regulated promoter that drives the expression of a regulatory gene. The minimal components therefore consist of a bacterial promoter, regulatory sites for control of the promoter, an mRNA leader sequence containing

translational initiation sequences, the structural gene for the desired regulatory protein, and a transcriptional terminator sequence. For genetic isolation, it is advisable to also include transcriptional termination sequences upstream from the regulated promoter. Fabrication consists of choosing the sequences to be used and assembling the sequences. The modules pertinent to the clock and toggle switch experiments are presented in Table 1. A much more extensive registry of parts for synthetic biology constructions, including parts developed by over 40 laboratories (including our own) is the registry of BioBricks (<http://parts.mit.edu>). This registry contains a large variety of promoter, operator, mRNA leader, and terminator elements, as well as a variety of structural genes encoding transcription factors and reporters. Standard cloning methods can be used to introduce any element from the biobricks collection into our vectors, by either performing a blunt end ligation or using PCR to add appropriate sites to the biobrick. We are currently developing a modified series of landing pad vectors that are able to directly accept any Biobrick fragment or construct. Although cloning from BioBricks or other sources is an appropriate way to build modules, we have found that in many cases, simply cloning annealed long oligonucleotides of desired sequence is the most direct way to build modules.

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Figure 1. Basic circuit topology of the synthetic genetic clock

The activator module (left) consists of a promoter, P_A , that drives the expression of activator, A . The repressor module (right) consists of a promoter, P_R , that drives the expression of repressor, R . Repressor blocks transcription from P_A .

Figure 2. Structure of the synthetic genetic clock

The activator module (left) consists of a modified version of the *glnA* promoter, driving the expression of *glnG* (*ntrC*). Light blue boxes in the *glnA* control region signify the *glnAp2* enhancer, dark blue boxes signify “perfect” *lac* operator sites, and grey boxes signify the *glnAp2* governor sites. The relative positions of the *glnAp1* and *glnAp2* transcription start sites are shown by bent arrows. The product of *glnG* (*ntrC*), NRI, is converted to its active, phosphorylated, form by NRII2302, which is provided in excess from a plasmid (not depicted). NRI~P increases its own expression by binding to the enhancer, whereupon it represses *glnAp1* and activates *glnAp2*. The repressor module (right) consists of the natural *glnK* promoter and translation initiation region fused to the *lacI* structural gene. The *glnK* promoter is associated with a weak enhancer (signified by a light blue box and a stippled light blue box). The product of the repressor module, LacI, blocks transcription from the activator module, and from the native *lacZYA* operon (bottom). Repression of the *lacZYA* operon is due to repressor interaction with the three operators of this operon (dark blue boxes), as indicated. The product of *lacZ*, β-galactosidase, serves as a reporter for oscillatory behavior.

Figure 3. The activator module is an N-IMPLIES gate with positive feedback

When in cells containing wild-type natural components of the Ntr system except for NRI, containing the activator module to provide wild-type NRI, containing the wild-type *lacI* gene, and containing a fusion of *lacZ* to the *glnK* promoter, the activator module provides an N-IMPLIES logic gate for the regulation of β-galactosidase by ammonia and IPTG.

Figure 4. The activator module can function as a toggle switch

The basic circuit topology for the toggle switch is shown at top. The activator module produces activator that drives expression of GS and the activator module. LacI is produced from the natural chromosomal *lacI* gene, and represses the activator module as well as the natural *lacZYA* (not depicted). At bottom, typical results for *lacZ* expression (left), and glutamine synthetase expression (right) are shown for induced (dot) and naive (+) cultures. The overlapping curves for the *lacZ* expression data show that the steady state was reached; the GS data shows hysteresis of the activator module. An intuitive explanation for this hysteresis is as follows: When the level of NRI~P is high, as in the induced culture, it is difficult for repressor to get control of the system, and thus repression is only achieved when the IPTG concentration is low. In the naive cell, the level of NRI~P is very low and thus it is considerably easier for repressor to get control of the system. Thus, in naive cells, the system stays repressed even at fairly high concentrations of IPTG.

Figure 5. The repressor module functions as an OR logic gate

When the repressor module is present in cells containing a mutation in the natural *lacI* gene, and with otherwise wild-type Ntr system and *lacZYA*, it provides OR gate function with regard to ammonia and IPTG for expression of *lacZYA*.

Figure 6. Results of clock experiments using a standard laboratory continuous culture device
The activator module used in the experiments is depicted at top left. The experiments were conducted as described in text, with manual control of the nutrient pump to maintain stable culture turbidity. At top right, results are shown from an experiment where OD₆₀₀ and β -galactosidase were monitored. At bottom left, another experiment where glutamine synthetase was also measured is shown, for clarity the OD₆₀₀ data is not shown for this experiment. As predicted by the model, the expression of glutamine synthetase and β -galactosidase was out of phase. At bottom right, the phase diagram for the β -galactosidase and glutamine synthetase expression data is shown. Note that if the system contained a perfect oscillator, the phase diagram would reveal a stable orbit instead of spiraling inward to a steady state.

Figure 7. Schematic diagram for a home-made turbidostat
For details and further description, see text.

Figure 8. Close-up view of the reactor for a home-made turbidostat
For explanation, see text.

Figure 9. Results of a clock experiment employing an automated system and miniaturized β -galactosidase assay.

Figure 10. The pStep0 plasmids that can be used to create new landing pads

The vector backbone consists of the EcoRI-SapI fragment of pBR322 containing the origin of replication (triangle) and ampicillin resistance gene (AB0). The plasmids contain a linker with multiple unique sites in either of two sequences, denoted “t” and “c”.

Figure 11. The pStep1-pStep4 plasmids, that can be used to place genetic modules onto the *E. coli* chromosome

For the pStep1 plasmids, T signifies the target for recombination with the chromosome, P1 signifies a module promoter, and lollipop symbols signify transcriptional terminators. The selective marker for pStep1 recombination is AB1. For the pStep2 plasmids, AB1 fragments constitute the target for recombination with the chromosome, P2 signifies a module promoter, and the selective marker for recombination is AB2. For the pStep3 plasmids, AB2 fragments constitute the target for recombination with the chromosome, P3 signifies a module promoter, and the selective marker for recombination is AB1. For the pStep4 plasmids, AB1 fragments constitute the target for recombination with the chromosome, P4 signifies a module promoter, and AB2 provides the selective marker for recombination. Note that the “t” and “c” series of plasmids differ in the arrangement of internal sites of the linker. For all plasmids, AB0 is the ampicillin resistance gene, containing the PstI site that may be used for plasmid linearization.

Figure 12. The sequence of nesting that occurs upon successive use of the pStep1-pStep4 plasmids for integration of modules into the chromosome

Symbols are as in Fig 11; G1-G4 depict structural genes within the modules, expressed from module promoters P1-P4. Recursive use of the pStep1-pStep4 plasmids to modify a chromosomal site results in side-by-side integration of modules on the chromosome, separated by transcriptional terminators.

Table 1

Useful strains, modules, and plasmids

Bacterial Strains for Clock and Toggle Switch Experiments				
Strain	Relevant Genotype	Source or Reference		
3.300	<i>lacI</i>	CGSC ¹		
3.300LG	<i>lacI glnL glnG</i>	(Atkinson et al, 2003)		
3.300LG-Act	3.300, but <i>rbs::glnApOG6 ...gen^r</i>	(Atkinson et al, 2003)		
NC12	3.300-Act, but <i>glnK::glnKp-lacI2...cam^r</i>	(Atkinson et al, 2003)		
NC12/p3Y15	NC12, but containing p3Y15 (<i>glnL2302</i>)	(Atkinson et al, 2003)		
TE2680	<i>recD::Tn10</i>	(Elliott, 1992)		
3.300-Rep	3.300, but <i>glnK::glnKplacI2...cam^r</i>	(Atkinson et al, 2003)		
M7044	<i>lacY</i>	CGSC ¹		
TS1	M7044, but <i>rbs::glnApOG6...gen^r</i>			

Landing pads				
plasmid	cloning	linearization	selection	location
pDK11	EcoRV (blunt)	PstI	cam	<i>glnK</i>
pRBS	(PstI, SalI, XbaI, SacI)	NotI, SpeI	gent	<i>rbsK</i>
pStep1t-AraBk	(Fig 7)	PstI	kan	<i>araB</i>
pStep1t-MalPk	(Fig 7)	PstI	kan	<i>malP</i>
pStep1t-FucKk	(Fig 7)	PstI	kan	<i>fucK</i>
pStep1t-MalPc	(Fig 7)	PstI	cam	<i>malP</i>
pStep1-MalQc	(Fig 7)	PstI	cam	<i>malQ</i>
pStep1t-FucKc	(Fig 7)	PstI	cam	<i>fucK</i>
pStep2-kan	(Fig 7)	PstI	cam	<i>kan</i>
² pStep3-cam	(Fig 7)			
² pStep4-kan	(Fig 7)			

Modules and plasmids		
Name	Function	Feature
<i>rbs::glnApOG6</i>	Activator module	<i>glnAp</i> with proximal and distal <i>lacOp</i> driving expression of <i>glnG</i>
<i>glnK::glnKplacI2</i>	Repressor module	<i>glnKp</i> driving expression of <i>lacI</i>
p3Y15	provides NR12302	<i>glnL2302</i> driven from its natural promoter, derived from pBR233 (Atkinson and Ninfa, 2003).

¹ coli genetic stock center: <http://cgsc.biology.yale.edu/>² under development.