Rapid and tunable post-translational coupling of genetic circuits

Arthur Prindle1*, Jangir Selimkhanov1*, Howard Li1, Ivan Razinkov1, Lev S. Tsimring2 & Jeff Hasty1,2,3

One promise of synthetic biology is the creation of genetic circuitry that enables the execution of logical programming in living cells. Such ‘wet programming’ is positioned to transform a wide and diverse swathe of biotechnology ranging from therapeutics and diagnostics to water treatment strategies. Although progress in the development of a library of genetic modules continues apace1–4, a major challenge for their integration into larger circuits is the generation of sufficiently fast and precise communication between modules5–8. An attractive approach is to integrate engineered circuits with host processes that facilitate robust cellular signalling9. In this context, recent studies have demonstrated that bacterial protein degradation can trigger a precise response to stress by overloading a limited supply of intracellular proteases10–12. Here we use protease competition to engineer rapid and tunable coupling of genetic circuits across multiple spatial and temporal scales. We characterize coupling delay times that are more than an order of magnitude faster than standard transcription-factor-based coupling methods (less than 1 min compared with 20–40 min) and demonstrate tunability through manipulation of the linker between the protein and its degradation tag. We use this mechanism as a platform to couple genetic clocks at the intracellular and colony level, then synchronize the multi-colony dynamics to reduce variability in both clocks. We show how the coupled clock network can be used to encode independent environmental inputs into a single time series output, thus enabling frequency multiplexing (information transmitted on a common channel by distinct frequencies) in a genetic circuit context. Our results establish a general framework for the rapid and tunable coupling of genetic circuits through the use of native ‘queueing’ processes such as competitive protein degradation.

To engineer rapid coupling between synthetic genetic modules, we developed a post-translational coupling platform that operates via shared degradation by the ClpXP protease (Fig. 1a). In this scheme, all LAA-tagged components13 are dynamically linked through competition for a linker between the protein and its degradation tag. We use this mechanism as a platform to couple genetic clocks at the intracellular and colony level, then synchronize the multi-colony dynamics to reduce variability in both clocks. We show how the coupled clock network can be used to encode independent environmental inputs into a single time series output, thus enabling frequency multiplexing (information transmitted on a common channel by distinct frequencies) in a genetic circuit context. Our results establish a general framework for the rapid and tunable coupling of genetic circuits through the use of native ‘queueing’ processes such as competitive protein degradation.

We systematically explored the coupling mechanism by driving a constitutive module with a quorum-sensing clock (Fig. 1c). As the pacemaker, the quorum clock generates density-dependent synchronous oscillations at the colony level via acyl-homoserine lactone (AHL), a small molecule capable of synchronizing cellular behaviour across distances up to 100 μm14. Using microfluidic devices15 we observed the colony-level expression of the constitutive module, and found that oscillating expression was synchronized to the quorum clock (Fig. 1c, top right). We then constructed a library of degradation tags by adding a series of variable-length spacer regions between the downstream protein and its degradation tag. Spacer regions contained between one and five copies of the amino acid sequence ‘Thr-Ser’ (TS) and their effects on offset time compared to that of a previously published alternative degradation tag (Extended Data Fig. 1b–f). Although all spacer sequences produced synchronous activation dynamics, the degradation dynamics of the downstream module were offset depending on the length of the linker sequence, where longer linkers produced greater GFP–CFP offset time (Fig. 1c, bottom). Thus, our ClpXP coupling platform rapidly links genetic modules through shared degradation and permits tuning the strength and timing of coupling by changing the degradation kinetics of individual modules.

To engineer coupling between genetic modules capable of generating their own dynamics, we designed a circuit containing the quorum clock and a variant of a previously described intracellular clock (Fig. 2a)16. This intracellular clock variant based on the Plac/ara-1 promoter retains the fast dynamics and simple genetic architecture of the published version that uses the PlacO-1 promoter, yet its period is tunable by both isopropyl-β-D-1-thiogalactopyranoside (IPTG) and arabinose in the presence of chromosomal araC. We first used small microfluidic devices (100 cells) and observed fast and asynchronous intracellular clock oscillations without quorum clock contribution, as the quorum clock requires a critical colony size to function (Supplementary Video 1). In larger devices (5,000 cells), we observed a transition from asynchronous oscillations to identical intracellular and quorum clock oscillations as the population grew larger (Fig. 2b and Supplementary Video 2). In the case of the larger population, the substrate load on ClpXP during the quorum clock pulse is sufficient to shift the intracellular clock out of its oscillatory regime, enabling complete linkage between the two clocks despite their vastly different spatial and temporal scales. Thus, despite lacking a mode of cell–cell communication itself, the intracellular clock is effectively synchronized at the colony level through ClpXP-mediated coupling with the quorum clock.

We found that changing the intracellular clock period of individual cells indirectly tuned the quorum clock period, as IPTG values associated with longer intracellular clock periods inversely produced shorter quorum clock periods (Fig. 2c). We developed a computational model of the oscillator network involving a form of load-mediated pulse frequency modulation to explain this effect (Fig. 2d–f). Between coupled pulses, the intracellular clock accelerates the quorum pulse onset through load-mediated decreases in the degradation rate of LuxI, since larger intracellular...
**Figure 1** A rapid post-translational coupling platform based on shared degradation. a. We measured the delays associated with module-module coordination by ClpXP degradation (1 ± 1 min (± s.e.m.), represented by purple arrow) and input–output response through transcription and translation (31 ± 5 min) in a single experiment by inducing (red arrow) the lux promoter and tracking the response of superfolder GFP (sfGFP)-LAA (lux promoter, black arrow) and CFP-LAA (P_lac/ara-1 promoter, black arrow) in single cells (right panel, 55 gray cell trajectory pairs with 3 representative pairs highlighted). b. Rapid (<2 min, our experimental time-step) induction of protein degradation by externally provided H₂O₂ produces reversible changes in ClpXP load in response to obstruction of RsbY²⁴⁵. c. To use post-translational coupling to drive downstream modules, we linked a quorum clock to a constitutively expressed fluorescent protein via the addition of identical LAA tags. With identical degradation tags, the constitutive module couples tightly to the quorum pacemaker. The addition of a variable-length linker (Thr-Ser (TS) repeats) before the degradation tag phase-shifts the degradation dynamics, where longer linkers produced longer delays. Error bars indicate s.d. of offset time, centred at the mean (50–200 cells for each TS-linker length). a.u., arbitrary units.

**Figure 2** Post-translationally linked genetic clocks at multiple scales. a. The network is composed of coupled intracellular²⁷ and quorum clocks²⁷. The intracellular clock oscillates as a result of delayed negative feedback on its own promoter and its period is tunable by IPTG and arabinose. Quorum clock oscillations are tunable by media flow rate and are synchronized via AHL at the colony level. b. The coupled intracellular quorum clock system oscillates asynchronously in small populations and transitions to synchronized oscillations in larger populations once the quorum clock fires. Despite lacking a mode of cell–cell communication itself, the coefficient of variation (CV) of the intracellular clock drops markedly through host-linked coupling with the quorum clock (bottom, data from 28 single-cell traces). c. IPTG reduces the intracellular clock period in small cell populations without the quorum clock (blue) and increases the coupled period in larger populations with the quorum clock (red). Each data point taken from 10–30 oscillatory peaks. Error bars indicate s.e.m. of the period, centred at the mean. d. In our computational model, load-mediated coupling allows the intracellular clock to modulate the quorum clock period via degradation coupling at ClpXP, since the intracellular clock continues oscillating between coupled pulses and accelerates the pulse onset. e. This adaptive form of pulse frequency modulation ensures that the pulse dynamics remain unchanged while the inter-pulse duration is adjusted (left, model; right, experimental; 6–9 oscillatory peaks). Inset shows the earlier onset of the coupled pulse due to the intracellular clock. Error bars indicate s.e.m. of relative quorum clock period. f. This mechanism also makes the coupled system more robust by enabling oscillation at higher media flow rates. NFB, negative-feedback oscillator; QS, quorum-sensing oscillator.
clock load produces higher levels of the AHL-synthase (Fig. 2e, left, and Extended Data Fig. 2a–c). During the coupled pulse, contributions of the intracellular clock leave the duration of the pulse itself unchanged (Fig. 2e, left (model) and right (experimental)). Linking the intracellular and quorum clocks through degradation also yielded an expansion in the oscillatory regime for the coupled system with respect to flow rate compared to the quorum clock alone (Fig. 2f). In this way, the intracellular clock continually excites the quorum clock to fire, enabling more robust function at higher external flow rates (Extended Data Fig. 3a–c).

With a platform for rapidly coupling genetic clocks at multiple scales, we sought to engineer a system capable of frequency-encoding information from both clocks into the multispectral time series of a single reporter (Fig. 3a). Here, the measured output of the intracellular clock reporter contains contributions from its own fast intracellular clock dynamics between slow quorum clock bursts (Supplementary Video 3). As the range of natural periods for the faster $\beta_{lac}/ara-1$ intracellular clock is fully separated from the slower quorum clock16,19, both IPTG and arabinose and flow rate inputs can be encoded into frequency-modulated oscillations in the time domain and independently extracted by Fourier transform. Thus, the measurement of a single clock history reveals the activities both underlying clock networks.

We began by characterizing the frequency response curves for both the intracellular and quorum clocks in isolation, finding ranges of 7–25 min and 55–95 min, respectively, when sweeping IPTG and arabinose, and flow rate inputs (Fig. 3b, top (intracellular clock in araC+ strain) and bottom (quorum clock, original study data)17). We then measured trajectories taken from the coupled clock system and extracted the frequency components of both clocks by Fourier transform30 (Fig. 3c and Methods Summary). In sweeping IPTG and arabinose inducers, we found the frequency response of the intracellular clock contribution to the multispectral reporter to be unchanged by the inclusion of the quorum clock since the intracellular frequency response to IPTG and arabinose was equivalent to the isolated clock (Fig. 3d, top (coupled), and Fig. 3b, top (isolated)). We then swept flow rates at three fixed inducer levels, and found distinct response curves for the quorum clock contribution to the multispectral reporter shifted in accordance with our model for ClpXP-mediated frequency modulation by the intracellular clock (Fig. 3d, bottom). Thus, to decode a given pair of IPTG and arabinose, and flow rate inputs, we first recover the intracellular clock frequency as a measure of IPTG and arabinose and then use the corresponding quorum clock response curve to measure flow rate.

To extend rapid coupling to greater spatial scales, we added a genetic $H_2O_2$ signalling21 cassette to the network and observed synchronization at the multi-colony level (Fig. 4a and Supplementary Video 4). In conducting these experiments, we also observed $H_2O_2$-mediated interaction between the native stress response network and our synthetic circuit at ClpXP (Fig. 4b). In the original design, $H_2O_2$ synchronized quorum clock oscillations by transcriptional upregulation of the lux promoter via the aerobic response control system ArcAB22. In addition to transcriptional increase (Fig. 4c, top), we found an increase in the apparent degradation rate with $H_2O_2$ (Fig. 4c, bottom, and Extended Data Fig. 4a, b), consistent with increased ClpXP activity in response to externally provided $H_2O_2$. The coupled increases in transcriptional output and effective ClpXP degradation rate in response to $H_2O_2$ also tightens the period distribution at the multi-colony level by mitigating the effects of period variation in an individual colony (Fig. 4c, top, and Extended Data Fig. 4c, d).

Engineering synthetic circuits composed of interacting modules is an ongoing effort31 that has generally relied on transcription and translation, with less attention paid to post-translational coupling mechanisms2.
Figure 4 | Post-translational coupling at the multi-colony level. a, At the multi-colony level, interaction of H_2O_2 generated by redox signalling with the cellular stress response network synchronizes quorum clock oscillations between colonies. Traces taken from 10 separate colonies across the array. b, Host-linked oscillations change distinct aspects of the waveform in response to H_2O_2 produced by the enzymatic activity of NDH (NADH dehydrogenase II). With H_2O_2, oscillations have larger amplitudes and steeper downslopes, revealing increases in both transcription and degradation produced by the interaction of the synthetic clock network with the native stress response. Dark lines indicate the means of all trajectories. c, H_2O_2 increases the oscillatory amplitude while decreasing the required degradation time, revealing an increase in ClpXP activity. This increase in ClpXP capacity in response to H_2O_2 serves to mitigate the effects of transcriptional noise by minimizing the effects of amplitude variation on the period, resulting in a tightening of the period distribution with H_2O_2 (model, Extended Data Fig. 4c, d).

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

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Extended Data Figure 1 | Increasing the length of the TS linker sequence results in increasing downstream module degradation delay. a, Detailed breakdown of single fluorescent trajectory analysis. Peaks are identified in red, troughs in green, upslope 10% points in purple and downslope 10% points in dark grey. The two period measurements are peak to peak and the time between two successive 10% upslope points. b, Top, sfGFP does not show bleed-over into the CFP fluorescence channel. Induction of sfGFP with 10 nM acyl-homoserine lactone (AHL, dashed line) showed increase in fluorescence of sfGFP, which was not detected in the CFP channel. Bottom, the use of the published AAV degradation tag shows delay in the downstream module degradation of 15 min. c, Without the TS linker sequence, there is very little delay in downstream module degradation. d, Single TS linker sequence results in 10 min delay. e, Double TS linker sequence results in 16 min delay, similar to that of AAV degradation sequence. f, 5-TS linker sequence results in 25 min delay (data shown in c–f were used to generate Fig. 1c).
Extended Data Figure 2 | Cell–cell communication by AHL reduces variability in the quorum clock. 

**a,** Individual ‘leader’ cells show early activation of quorum clock proteins relative to the mean population response. 

**b,** In a two-cell simulation, cells 1 and 2 start out unlinked with slightly different constitutive production of AiiA and LuxI. At $t = 100$ min the two cells are linked through external AHL in the media, showing the cell with slower dynamics (cell 2) linking up to cell 1 with shorter periods. 

**c,** Cells 1 and 2 start out unlinked with cell 1 including intracellular clock dynamics (green) that result in higher frequency oscillations in cell 1. When the cells are linked ($t = 100$), the slower cell 2, without the intracellular clock, links on to the faster cell through external AHL communication between the cells. 

**d,** Trajectories of 20 cells (different colour traces) with noisy constitutive production at lux promoter synchronize when their external AHL pool is mixed at $t = 400$ min. Mean trajectory is shown in black. 

**e,** Period variability after cell synching (red) is lower than in individual cells (blue). QS, quorum-sensing oscillator.
Extended Data Figure 3 | The intracellular clock increases robustness in the coupled oscillator system by reducing the period of the quorum clock. 

a, Removal of IPTG, which increases intracellular clock strength, leads to more regular oscillations (experimental). b, The decrease in variability of the inter-pulse time of the coupled oscillator without IPTG suggests that the intracellular clock plays an important role in the inter-pulse dynamics (experimental). c, At very high flow rates, the quorum clock oscillates irregularly. Tuning up the intracellular clock reduces the quorum clock period, restoring regular oscillations and allowing for global level synchronization between colonies due to H₂O₂ biopixel coupling. Genetic addition of the intracellular clock (0.1 mM IPTG) helps synchronize the quorum clock at high flows (430 μm s⁻¹). Increasing the strength of the intracellular clock with removal of IPTG further enhances H₂O₂ inter-colony synchronization (experimental, black lines indicate the mean of experimental races).
Extended Data Figure 4  | H$_2$O$_2$ increases the degradation rate by ClpXP, and this in combination with transcriptional increase at the lux promoter decreases variability in the oscillator period.  

**a**, There is a significant decrease in the degradation time due to H$_2$O$_2$ (experimental).  

**b**, Decrease in the degradation time due to H$_2$O$_2$ is due to effective increase in ClpXP degradation rate (experimental).  

**c**, H$_2$O$_2$ activation of lux promoter alone would only increase the amplitude of quorum clock oscillations. Similarly, H$_2$O$_2$-dependent increase in ClpXP activity results only in steeper degradation and longer inter-pulse duration. Combination of the two effects leads to increase in amplitude and decrease in inter-pulse duration, which matches experiments (model).  

**d**, Individually, the two H$_2$O$_2$ effects do little to lower the quorum clock period CV, which is reduced when both are present (model).