

Supplemental Information: Polyphasic feedback enables tunable cellular timers

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Model Parameter Definitions

Parameter	Description	Units
x_0	Initial regulator concentration	Concentration (Molecules / Cell Volume)
x_F	Threshold regulatory concentration for differentiation	Concentration (Molecules / Cell Volume)
γ	Dilution/degradation rate constant	1/Time
K	Concentration of x required for half-maximal x promoter activation	Concentration (Molecules / Cell Volume)
N	Number of circuit pulses during deferral period	Dimensionless
β_{OL}	Open loop circuit promoter strength (Maximal production rate from promoter)	Concentration / Time
β_C	Continuous feedback circuit promoter strength (Maximal production rate from promoter)	1 / Time
β_P	Polyphasic feedback circuit promoter strength (Maximal production rate from promoter)	Dimensionless (see text)
τ_C	Deferral time for continuous circuit (Time for x to reach x_F starting from x_0)	Time
τ_P	Deferral time for continuous circuit (Time for x to reach x_F starting from x_0)	Time
S_C	Sensitivity of deferral time to feedback strength for continuous circuit	Time ²
S_P	Sensitivity of deferral time to feedback strength for polyphasic circuit	Time ² (see text)
T	Period for circuit pulsing (time between the start of successive pulses of activation)	Time
n	Pulse period index	Dimensionless

Full Description of Polyphasic Positive Feedback Circuit Model.

Equation (S1) is an explicit phase-by-phase description in the n th period of the polyphasic feedback circuit that also incorporates saturating feedback with saturation constant K .

$$\frac{dx(t)}{dt} = -\gamma x(t) + \begin{cases} 0, & (n-1)T \leq t \leq nT - \Delta t \quad \text{phase 1 \& 2 of period } n \\ \frac{\beta_P}{\Delta t} \frac{x(nT - \Delta t)}{K + x(nT - \Delta t)}, & nT - \Delta t < t < nT \quad \text{phase 3 of period } n \end{cases} \quad (\text{S1})$$

Here, $x(nT - \Delta t)$ denotes $x(t)$ evaluated at time $nT - \Delta t$.

Figure Simulation Details

Here we describe the numerical simulations used in the main figures. All simulations were performed using MATLAB's built in ode45 integrator.

The time trace in Figure 1B is obtained by integrating Equation (1). Parameters were chosen so that each cell cycle takes unit time and $x(t)$ crosses the threshold of $x(t) = 100$ at 5 cell cycles.

The time trace in Figure 1C is obtained by integrating the equation $\frac{dx(t)}{dt} = -\gamma x(t) + \beta_C \frac{x(t)}{(K+x(t))}$, a more realistic version of Equation (3) incorporating saturating feedback, with $K = 100$. Parameters were chosen so that each cell cycle takes unit time, and so that $x(t)$ crosses the threshold of $x(t) = 100$ at 5 cell cycles.

The time trace in Figure 1D is obtained by integrating equation (S1), a generalization of Equation (5) incorporating saturating feedback, with $K = 100$. We used the unit time step of the ode45 integrator as Δt to approximate an extremely brief pulse. Parameters were chosen so that each cell cycle takes unit time and so that $x(t)$ crosses the threshold of $x = 100$ at 5 cell cycles.

The tuning curves in Figure 1B-D (lower panels) were generated by simulating the respective systems for several β values and recording the time for x to reach a threshold of $x(t) = 100$ in each case. All other parameters in tuning curve simulations are identical to those used for time trace simulations.

Pulsing Alone Does Not Increase The Tunability of Positive Feedback.

In order to disentangle the contributions of pulsing and phasing from the performance of the polyphasic feedback circuit, consider a pulsed, but not polyphasic, positive feedback circuit where the production rate during the pulse is a function of the instantaneous value of $x(t)$.

$$\frac{dx(t)}{dt} = -\gamma x(t) + \begin{cases} 0, & (n-1)T \leq t \leq nT - \Delta t & \text{phase 1 \& 2 of period } n \\ \frac{\beta_C}{\Delta t} x(t), & nT - \Delta t < t < nT & \text{phase 3 of period } n \end{cases} \quad (\text{S2})$$

We can solve this model one phase at a time as in the polyphasic case. The solution is

$$x(nT) = x_0 e^{n\beta_C} e^{-\gamma nT} \quad (\text{S3})$$

The exponential growth rate of $x(t)$ is linearly dependent on β_C , just like the non-pulsed continuous feedback case, and in contrast to the logarithmic dependence on β_P of the polyphasic feedback case.

Polyphasic and Continuous Feedback are Equivalent at High Pulse Frequencies.

In this section we study how circuit deferral times depend on feedback strength.

Deferral time is the time required for the regulator, x , to start from an initial concentration, x_0 , and accumulate to a threshold value, x_F . We denote the deferral times of continuous and polyphasic

positive feedback circuits as τ_C and τ_P , respectively. We assume that the polyphasic circuit pulses N times, with period $T = \tau_P/N$ (Figure S1A).

To compare the two circuits we compute the sensitivity of their deferral times (τ_C and τ_P) to their respective feedback strengths (β_C and β_P). We perform this calculation analytically for non-saturating linear feedback, and numerically for saturating feedback.

We show that at high pulse frequencies ($\frac{1}{T} \gg 1$), with appropriately scaled feedback strength, the polyphasic system becomes identical to the continuous positive feedback system (Supplementary Figure 1B), with similar sensitivity to feedback strength.

We define the continuous circuit's sensitivity, S_C , as the rate of change of deferral time with respect to changes in feedback strength, e.g. $S_C \equiv \partial\tau_C/\partial\beta_C$. Here, τ_C and β_C are related by $\tau_C = \frac{\log\frac{x_F}{x_0}}{\beta_C - \gamma}$. S_C is thus

$$S_C = -\frac{\log\left(\frac{x_F}{x_0}\right)}{(\beta_C - \gamma)^2} \quad (\text{S4})$$

We now define the polyphasic circuit's sensitivity, S_P , with the following observations:

1. To compare S_P with S_C they must have the same units. Although τ_P and τ_C both have units of time, β_P is dimensionless while β_C is in units of inverse time. To keep dimensions consistent, we compare β_C with β_P/T (For a given pulse frequency, T remains constant as β_P changes).

The following argument justifies this comparison, using the non-saturating models. Consider polyphasic and continuous circuits, each tuned to the same deferral time. During each period T , each circuit must accumulate the same amount of $x(t)$. Equating equations (4) and (6) from the main text for one period leads to the relation:

$$e^{(\beta_C - \gamma)T} = (1 + \beta_P)e^{-\gamma T} \quad (\text{S5})$$

At high pulse frequencies where $N \rightarrow \infty$ as τ_P remains constant, $T = \frac{\tau_P}{N} \rightarrow 0$, and the normalized feedback strengths are related through:

$$\beta_C = \frac{\beta_P}{T} \quad (\text{S6})$$

This relates the production rate of the continuous circuit, in units of inverse time, to an effective polyphasic production rate expressed in production per period of time.

2. In the limit of infinitely brief pulses, the polyphasic circuit's deferral time does not change continuously as we change β_p . Rather, because pulses occur with period T , the deferral time τ_p essentially changes in multiples of T . Let $\Delta\beta_p$ denote the minimum increase in β_p required to shift the threshold crossing time forward by one period.

We can now define the polyphasic sensitivity S_p as the minimum change in deferral time, e.g. a single period $\Delta\tau_p = T$, divided by the minimum required change in time-normalized feedback strength, β_p/T :

$$S_p = \frac{\Delta\tau_p}{\Delta\left(\frac{\beta_p}{T}\right)} = \frac{T}{\frac{\Delta\beta_p}{T}} \quad (\text{S7})$$

Sensitivity Equivalence in Non-Saturating Circuits : Analytic Demonstration

To calculate S_p we first calculate $\Delta\beta_p$. By solving equation (6) from the main text for β_p , we find that the minimum feedback strength required to cross threshold in N pulses is $\beta_{p,N} = \sqrt[N]{\frac{x_F}{x_0} e^{\gamma NT}} - 1$. To shift the threshold crossing time forward by one period the circuit will cross threshold in $N - 1$ pulses, so $\Delta\beta_p \equiv \beta_{p,N} - \beta_{p,N-1}$.

In the high pulse frequency limit, where $N \rightarrow \infty$ as $\tau_p = \tau_c$ remains constant, and recalling the definition for τ_c used in (S4), shows that $\lim_{N \rightarrow \infty} S_p = S_c$:

$$\lim_{N \rightarrow \infty} S_p = \lim_{N \rightarrow \infty} \frac{\left(\frac{\tau_p}{N}\right)^2}{N \sqrt{\frac{x_F}{x_0} e^{\gamma NT}} - N-1 \sqrt{\frac{x_F}{x_0} e^{\gamma(N-1)T}}} \rightarrow -\frac{\log\left(\frac{x_F}{x_0}\right)}{(\beta_c - \gamma)^2} = S_c \quad (\text{S8})$$

At high frequencies the polyphasic circuit becomes as sensitive to changes in feedback strength as the continuous positive feedback circuit. In the next section, we show the same point in the presence of saturating nonlinearity using a numerical calculation (Supplementary Figure 1B).

Sensitivity Equivalence in Saturating Circuits : Numerical Demonstration

We next investigated this difference in the saturating circuits described in the text. Recall that the continuous circuit dynamics, with saturation, are

$$\frac{dx(t)}{dt} = -\gamma x(t) + \beta_C \frac{x(t)}{K + x(t)} \quad (\text{S9})$$

The polyphasic circuit dynamics were simulated with the approximation of an infinitely short ($\Delta t \rightarrow 0$) ‘ δ -function’ pulse for phase 3:

$$\frac{dx(t)}{dt} = -\gamma x(t) + \begin{cases} 0, & (n-1)T \leq t < nT \quad \text{phase 1 \& 2 of period } n \\ \beta_P \frac{x(nT)}{K + x(nT)}, & t = nT \quad \text{phase 3 of period } n \end{cases} \quad (\text{S10})$$

For each circuit we computed the deferral time as the time to reach a value $x_F = 100$ starting from an initial condition of $x_0 = 1$. We set both circuits in a moderately saturating regime with $K = 100$.

To plot polyphasic sensitivity versus pulse frequency, we simulated circuits with increasing pulse frequencies but equal deferral times ($\tau_P = 5$ cell cycles). (Figure S1B). For each pulse frequency, which defines a corresponding T , we first found the smallest β_P value that caused the circuit to cross threshold in 5 cell cycles. We then computed S_P by numerically finding the smallest $\Delta\beta_P$ that shifts the threshold crossing time up by one period to 4 cell cycles. We then computed S_P as defined in equation (S7) (Solid circles). Finally, we computed the continuous circuit sensitivity S_C for a 5 cell cycle deferral time (dashed line). Consistent with analytic results for linear (non-saturating) feedback, in the high pulse frequency limit, S_P approaches S_C for saturating circuits as well.

Polyphasic Feedback is Analogous to Infrequently Compounding Interest.

The differences between polyphasic positive feedback and continuous positive feedback can be understood using an analogy to interest rates.

Consider a bank account with starting value v_0 . If the account bears interest with annual interest rate r , and interest compounds n times per year, then the value of the account after t years is

$$v(t) = v_0 \left(1 + \frac{r}{n}\right)^{\lfloor nt \rfloor} \quad (\text{S11})$$

In the limit of continuous compounding ($n \rightarrow \infty$), the value becomes

$$v(t) = v_0 e^{rt} \quad (\text{S12})$$

Compounding interest acts as a non-saturating positive feedback, and interest rate plays the role of feedback strength. These equations suggest that continuous linear positive feedback behaves like continuously compounding interest, while polyphasic positive feedback behaves like infrequently compounding interest since sampling and production occur in a periodic, discrete manner.

The response of these accounts to a change in interest rate mimics the response of positive feedback circuits to changes in feedback strength. At the low interest rates involved in typical financial transactions (on the order of 1% - 10%), the two accounts behave similarly, as is well known in finance. At extremely high interest rates (on the order of 100%, sometimes seen in predatory lending or during extreme financial instability), the two compounding schemes can behave very differently.

The high interest rate regime corresponds to the feedback strength relevant to biological timers. For example, in *Bacillus subtilis*, 'low threshold' and 'high-threshold' sporulation promoters differ by ~65-fold in their affinities for the master regulator Spo0A. The low threshold *skf* gene has a dissociation constant of ~26nM compared to ~1700nM for *spolIG* [S1]. To achieve a comparable ~65-fold increase in a bank account balance, from \$26 to \$1700 in 5 compounding events requires 130% interest. Using round numbers, a 100-fold increase in a bank account from \$10 to \$1000 in 5 compounding events requires an interest rate of approximately 150%.

To illustrate the differences in sensitivity, consider two bank accounts, which compound at different frequencies. Both start with a balance of \$1,000.00. One account compounds daily (approximating continuous positive feedback), while the other account compounds monthly (corresponding to polyphasic positive feedback). The interest rates of both accounts are set so that after 5 months each account ends up valued at \$100,000, to correspond to an assumed 100 fold change in regulator concentration. The daily compounding account thus earns ~3.1% interest each day, while the monthly compounding account earns ~151% interest each month. In months {0, 1, 2, 3, 4, 5}, both accounts have balances of {\$1000, \$2512, \$6310, \$15,849, \$39,811, \$100,000}.

Now assume that an interest rate shock suddenly doubles interest rates. The daily compounding account's interest rate is now ~6.2% and the monthly compounding account's interest rate is now ~302%.

In months {0, 1, 2, 3, 4, 5}, the daily compounding account compounding at 6.2% has balances of {\$1000, \$6319, \$37,685, \$231,340, \$1,420,162, \$8,718,109}.

In months {0, 1, 2, 3, 4, 5}, the monthly compounding account compounding at 302.4% has balances of {\$1000, \$4024, \$16,191, \$65,148, \$262,140, \$1,054,793}.

The 5 month balance of the daily compounding scheme is now ~\$8.7M, while the 5 month balance of the monthly compounding scheme is only ~\$1.0M.

The monthly compounding scheme is much less sensitive to interest rate shocks than the daily compounding scheme. This reduced sensitivity is analogous to the reduced sensitivity of polyphasic feedback to changes in feedback (promoter) strength discussed in the main text.

Adding Slow Phosphorylation Kinetics to an Open Loop Circuit Does Not Significantly Extend Timescales.

Consider an extension of the open loop model described by equation (1) of the main text. In this extended model, the protein exists in two states: unphosphorylated/inactive $x(t)$, and phosphorylated/active x_p . We denote total protein concentration by $x_T(t) = x(t) + x_p(t)$. For simplicity, $x(t)$ is phosphorylated to $x_p(t)$ with a constant (saturated) kinase rate constant of k_k . Conversely, $x_p(t)$ is dephosphorylated to $x(t)$ with a constant (saturated) phosphatase rate constant of k_p . All proteins are diluted and degraded with total rate constant γ .

In this model, can parameters be chosen so that $x_p(t)$ equilibrates at a time scale much longer than one cell cycle? Intuitively, one might suspect that very slow kinase and/or phosphatase rate constants would allow x_p to equilibrate slowly.

The dynamic equations are:

$$\begin{aligned}\frac{dx_T(t)}{dt} &= \beta - \gamma x_T(t) \\ \frac{dx(t)}{dt} &= -\gamma x(t) + \beta - k_k x(t) + k_p x_p(t) \\ \frac{dx_p(t)}{dt} &= -\gamma x_p(t) + k_k x(t) - k_p x_p(t)\end{aligned}\tag{S13}$$

We can solve for $x_p(t)$, assuming initial conditions $x_T(0) = x_p(0) = 0$. Defining $\Gamma = \gamma + k_k + k_p$ for convenience,

$$x_p(t) = k_k \frac{\beta}{\gamma} \left[\frac{1}{\Gamma} + \frac{\gamma}{\Gamma(\Gamma - \gamma)} e^{-\Gamma t} - \frac{1}{\Gamma - \gamma} e^{-\gamma t} \right]\tag{S14}$$

Note that the timescales in this expression are generally faster than or equal to γ , suggesting that the system cannot respond arbitrarily slowly. To check this inference explicitly, we performed numerical simulations (Figure S2), which demonstrate that in this circuit, even at extremely slow phosphorylation and/or dephosphorylation rates, x_p equilibrates no more slowly than ~ 2 -3 cell cycles, despite a decrease in rate constants over several orders of magnitude (plateau in equilibration times). Thus, addition of the phosphorylation degree of freedom does not circumvent the limitations imposed by

dilution of circuit components. See also related work by Zwicker et al in the context of the cyanobacterial circadian clock [S2].

Supplementary Figures

Figure S1

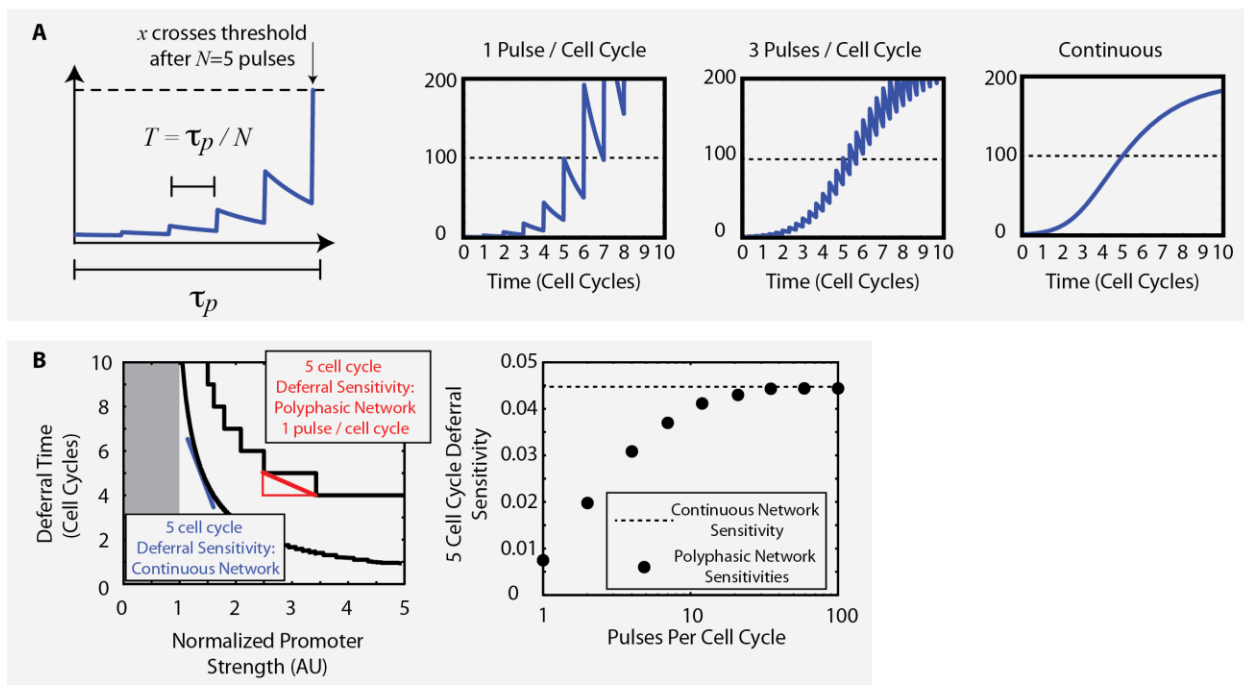


Figure S2

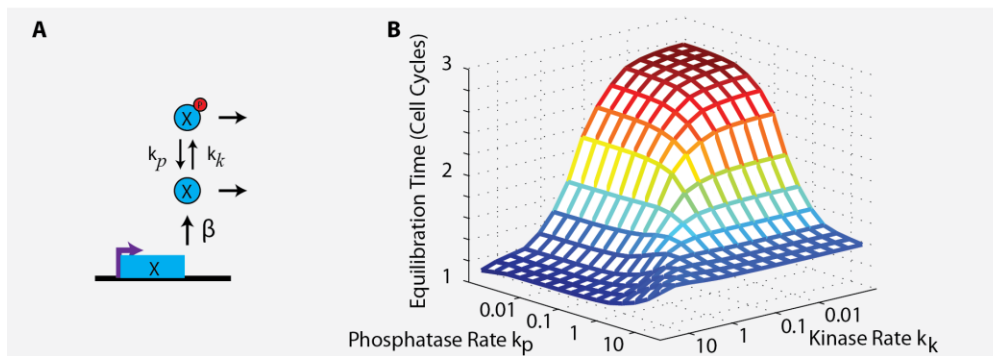


Figure S1. Polyphasic and continuous feedback circuits behave similarly in the limit of high pulse frequency. (A) (Left) Definition of quantities describing the polyphasic circuit. (Right) Plotted are three saturating networks with $K = 100$ tuned to cross threshold $x_F = 100$ at 5

cell cycles starting from $x_0 = 1$; (left) a polyphasic circuit with one pulse per cell cycle, (center) a polyphasic network with 3 pulses per cell cycle, and (right) a continuous circuit. As polyphasic pulse frequency increases, the circuit behaves more like its continuous counterpart. (B) Deferral sensitivities of polyphasic circuits approach that of the continuous circuit in the limit of high pulse frequency. (Left) Deferral sensitivity is defined as the change in deferral time caused by a change in feedback strength (promoter strength). Continuous network deferral sensitivity (blue) is defined as $S_C = \partial\tau_C/\partial\beta_C$ (Eq. S4). Polyphasic network deferral sensitivity (red) is defined as $S_P = \frac{\Delta\tau_P}{\Delta(\beta_P/T)}$ (Eq. S7). (Right) As pulse frequency increases, the deferral time sensitivity of polyphasic circuits (solid circles) increases, approaching that of continuous circuits (dashed line) in the high frequency limit.

Figure S2. Phosphorylation does not overcome the effects of dilution rate on equilibration time. (A) A simple model of an open loop circuit with phosphorylation (cf. Eq S13). Regulator $x(t)$ is constitutively produced in an inactive state, and is activated by phosphorylation to form $x_p(t)$. Both species are diluted/degraded with total rate constant γ as in all other models. (B) Slow phosphorylation rate constants have only a limited effect on deferral time. Plotted is the deferral time of the network of Eq S13. Starting at zero initial conditions, we define deferral time as the time in cell cycles required for $x_p(t)$ to reach $1 - e^{-1} \sim 63\%$ of steady state. We plotted this as a function of kinase and phosphatase reaction rates (k_k and k_p respectively), and observed that deferral times increase with slower reaction rates but plateau at a maximal value of ~ 2 -3 cell cycles.

Supplemental References

- S1. M. Fujita, J. E. Gonzalez-Pastor, R. Losick, *J Bacteriol* **187**, 1357 (Feb, 2005).
- S2. D. Zwicker, D. K. Lubensky, P. R. ten Wolde, *Proc Natl Acad Sci U S A* **107**, 22540 (Dec 28, 2010).