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SYNTHETIC BIOLOGY

Precision timing in a cell

A 16-year-old synthetic genetic circuit that produces gene-expression oscillations in bacterial cells has been given an upgrade, making it an exceptionally precise biological clock.

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Living cells keep track of time with exquisite precision, despite using molecular components that are subject to unavoidable random fluctuations, known as noise. For example, natural circadian clocks can track the time of day, even in single-celled cyanobacteria[1]. Such clocks have been selected over evolutionary timescales for their precision, and thus can be thought of as a literal embodiment of the biologist Richard Dawkins' 'blind watchmaker'[2] — his analogy for evolution's ability to produce systems with astonishing capabilities. However, evolution is not the only way to make a biological clock. The field of synthetic biology is based on designing artificial genetic circuits to implement new functions in living cells. Can a synthetic clock rival the precision of its naturally evolved counterparts? In a paper online in *Nature*, Potvin-Trottier *et al.*[3] demonstrate that even a relatively simple synthetic clock circuit can be astonishingly precise.

The starting point for the authors' work is a synthetic oscillating genetic circuit called the repressilator[4], now 16 years old. The repressilator, along with a contemporaneous synthetic toggle switch[5], showed that new genetic circuits could be designed from modular genetic elements and their behaviour analysed in living cells. More specifically, it showed that a totally synthetic circuit could generate dynamic oscillations in protein expression, making bacterial cells 'blink' on and off through periodic synthesis of a fluorescent reporter protein.

The repressilator uses a simple design, resembling a game of rock, paper, scissors. The key components are repressor proteins, which bind to specific DNA sequences adjacent to a target gene to inhibit gene expression. Three repressors are configured so that each one represses expression of the next in a cycle. One repressor also inhibits expression of a gene encoding the fluorescent reporter.

This configuration results in a negative-feedback loop, in which an increase in concentration of one repressor protein causes a decrease in the second, leading to an increase in the third, thereby decreasing the first. Mathematically, this circuit was predicted to generate limit cycles — a type of oscillation that is robust to perturbations and cannot 'damp out'. Nevertheless, because many relevant biochemical parameters were unknown, it was unclear whether the circuit would oscillate at all. The appearance of roughly periodic expression of the fluorescent reporter in individual cells was both reassuring and somewhat surprising. But the oscillations were quite noisy, varying in both their timing and amplitude.

Since then, researchers have designed a wide variety of synthetic oscillators[6] that incorporate alternative designs[7], coupling between cells[8] and other features[9,10]. But what limits the precision of a synthetic clock operating in a single cell remains unclear. On the one hand, incorporating additional feedback loops or other circuitry might help to tame the effects of noise. On the other, each additional component and interaction introduces an additional source of noise.

Motivated by this conundrum, Potvin-Trottier *et al.* revisited the repressilator to see whether it could be made more precise. The authors manipulated the genetic circuit and read out its behaviour in individual bacterial cells for more than 100 generations using a microfluidic device[11]. In this way, they meticulously identified and mitigated each source of noise in the circuit (Fig. 1).

First, they observed that some variability originated from poorly regulated replication of the circular DNA molecule (plasmid) that contained the fluorescent gene. This variation could be removed by integrating the reporter directly into the more tightly regulated repressilator plasmid that carried the genes for the three repressor proteins.

Second, to accelerate the oscillations, each repressor protein was originally engineered to undergo active degradation. However, this made repressor levels sensitive to variability in the cell's degradation machinery, and this effect was exacerbated by the many copies of the degradable fluorescent protein that were being produced. Lowering the number of copies of the reporter gene, or eliminating

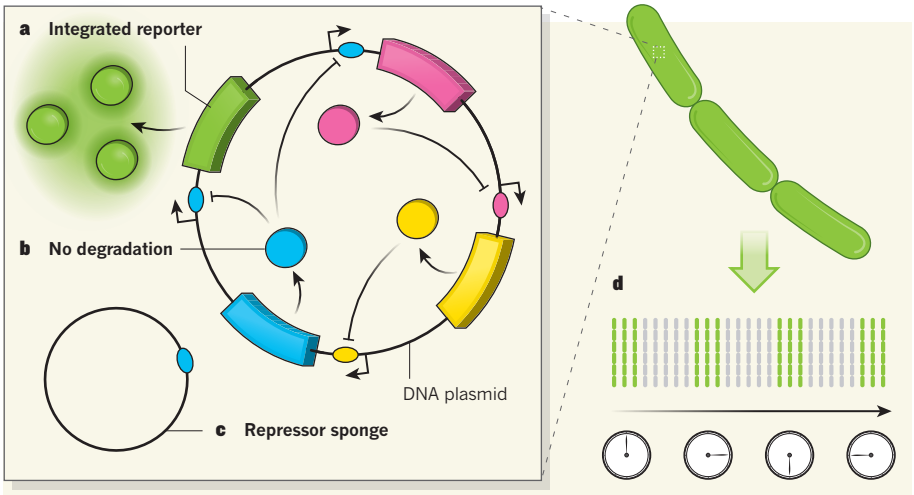

Figure 1 | The repressilator gets an upgrade. The represillator is a synthetic genetic circuit that produces periodic pulses of gene expression. It is based on a core set of three repressor proteins (pink, yellow and blue circles), which each bind to a DNA sequence adjacent to the gene encoding another repressor (oval binding sites for and rectangular genes encoding each repressor are colour coded). In this way, each protein represses the next. One of the repressors also inhibits production of a fluorescent protein (green). **a–c**, Potvin-Trottier *et al.*[3] made modifications to the original circuit that improved the precision of the repressilator. They included the fluorescent reporter gene on the same DNA plasmid as the repressor genes (**a**), prevented degradation of the proteins through the cell-degradation machinery (**b**) and introduced a 'DNA sponge' construct that contained binding sites for one particularly efficient repressor to raise the threshold at which expression of its target gene was reactivated (**c**). **d**, The improved repressilator oscillates with high precision, as shown by lines of cells growing in a microfluidic device that fluoresce green at regular time intervals (adapted from ref. 3).

active degradation entirely, strongly improved precision.

Finally, one of the repressors, TetR, has such a strong affinity for its DNA-binding site that its levels must decline below an extremely low threshold of around five proteins per cell before its target gene is re-expressed. This makes the timing of reactivation sensitive to the loss of just a few molecules, and therefore highly stochastic. To circumvent this effect, the authors increased the threshold by adding competing TetR-binding sites on a separate 'DNA sponge' plasmid. In the original design, this sponge role was serendipitously fulfilled by the reporter plasmid. Including this sponge (minus the reporter gene) further improved precision.

All told, in the most precise of Potvin-Trottier and colleagues' circuits, the standard deviation in period length was reduced from 35% of the mean to around 14%, with strikingly uniform pulse shapes and amplitudes observed. This repressilator generates a pulse of fluorescent-protein expression just once every 14 generations. Assuming a cell-cycle time of 1 hour, it would take around 7.5 days, or 180 cell cycles, for a colony of cells to accumulate a standard deviation of half a period of drift. This extraordinary precision means that even a large population of cells can remain in sync. In fact, the authors were able to visualize oscillation dynamics in a test-tube culture, and to track the history of oscillations in patterns of concentric fluorescent rings deposited as a repressilator colony grew outwards from the centre of a Petri dish. Evidently, precision does not necessarily demand circuit complexity and, in this case, even seems to benefit from minimalism.

The upgraded repressilator should provoke fresh questions. For example, cell growth rate directly affects the period of the oscillations, particularly in circuit variants in which repressors do not undergo active degradation. Is it possible to design clocks to oscillate independently of growth, without introducing additional variability?

Potvin-Trottier and colleagues' oscillator could enable dynamic analysis of natural gene circuits, by generating periodic perturbations of a gene of interest within cells. It could also become a module within larger synthetic circuits. For instance, in cell-based therapies the dynamics of drug delivery seems to have a major effect on drug specificity[12] — descendants of the upgraded repressilator could eventually enable periodic secretion of drug pulses in a human host.

The effects of noise are typically suppressed in electronic and mechanical systems. But for genetic circuits, noise remains a fact of life. That we can now design cells to operate with remarkable precision in the face of noise suggests that synthetic biologists are starting to become pretty good watchmakers, after all. ■

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