

# Diversity in the dynamical behaviour of a compartmentalized programmable biochemical oscillator

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***In vitro* compartmentalization of biochemical reaction networks is a crucial step towards engineering artificial cell-scale devices and systems. At this scale the dynamics of molecular systems becomes stochastic, which introduces several engineering challenges and opportunities. Here we study a programmable transcriptional oscillator system that is compartmentalized into microemulsion droplets with volumes between 33 fl and 16 pl. Simultaneous measurement of large populations of droplets reveals major variations in the amplitude, frequency and damping of the oscillations. Variability increases for smaller droplets and depends on the operating point of the oscillator. Rather than reflecting the stochastic kinetics of the chemical reaction network itself, the variability can be attributed to the statistical variation of reactant concentrations created during their partitioning into droplets. We anticipate that robustness to partitioning variability will be a critical challenge for engineering cell-scale systems, and that highly parallel time-series acquisition from microemulsion droplets will become a key tool for characterization of stochastic circuit function.**

Biological networks can perform highly sophisticated tasks such as signal processing, computation and the orchestration of molecular processes in time and space. In the past decade a variety of increasingly complex artificial, programmable molecular circuits have been demonstrated *in vitro*. On a fundamental level, such systems can be used to prototype and analyse subsystems of more-complicated naturally occurring circuits, and thus serve as a training ground for understanding biological complexity. Beyond biology, *in vitro* molecular systems offer considerable design flexibility using a limited number of well-characterized components<sup>1–3</sup>. Thus, they constitute an ideal platform for developing nanosystems that operate in a cell-free environment and exploit attractive features of cellular machinery, such as the ability to replicate, self-assemble and compute at the nanoscale.

An important step towards engineering compact biomimetic systems is the encapsulation of biochemical circuitry within cell-like microcompartments—the creation of programmable ‘artificial cells’ or ‘protocells’. However, compartmentalization can profoundly influence biochemical reaction kinetics<sup>4,5</sup>. In particular, typical concentrations of molecules in biomolecular circuits are in the nanomolar range, which for cell-sized reaction containers means that some molecular species are present only at very small copy numbers. A prominent consequence of this is the appearance of biochemical ‘noise’, often attributed to the inherent stochasticity of chemical reactions<sup>6</sup>. Understanding the sources and propagation of noise and variability in artificial biochemical systems encapsulated in microcompartments is necessary to provide a foundation for engineering molecular systems that are robust to stochasticity, or that exploit the randomness to advantage. Furthermore, because biological cells face many of the same challenges and opportunities, our understanding of biological systems can be informed

by, and can provide insight into, engineering principles for molecular systems at this scale.

Cell-free gene expression in lipid bilayer vesicles has been studied in the past, but functional encapsulation of complex mixtures of biochemicals, such as the transcription/translation machinery, remains challenging<sup>7,8</sup>. A technologically more-developed approach employs water-in-oil emulsion droplets as reaction containers<sup>5,9,10</sup>, with applications that include single-molecule enzymology<sup>11</sup>, emulsion polymerase chain reaction (PCR)<sup>12</sup> and *in vitro* evolution experiments for the selection of ribozymes or functional proteins<sup>13,14</sup>. However, thus far the influence of micron-scale encapsulation on *in vitro* biochemical networks with more-complex, far from equilibrium, dynamical behaviour has not been investigated systematically.

Previously, compartmentalization of an inorganic chemical dynamical process was demonstrated with the Belousov–Zhabotinsky reaction, where the emergence of oscillations and spatial patterns was studied in microemulsion droplets<sup>15</sup>. As inorganic chemical oscillators operate at much higher concentrations than typical biochemical systems, fluctuations and ‘small-number effects’ were, with few exceptions<sup>16,17</sup>, typically not observed. Recently, a range of synthetic biochemical oscillators have been demonstrated successfully both *in vivo*<sup>18–22</sup> and *in vitro*<sup>3,23–25</sup>. Owing to their important role in the orchestration of biological processes<sup>26,27</sup>, there is considerable interest in the robustness of naturally occurring biochemical clocks with respect to molecular noise<sup>27–29</sup> or temperature fluctuations<sup>30</sup>. So far, synthetic *in vitro* biochemical oscillators have been studied only in bulk reactions<sup>3,24,25,31</sup>, or encapsulated into emulsion droplets too large to result in considerable dynamic variability<sup>32</sup>.

Here, we demonstrate and characterize compartmentalization of a synthetic biochemical clock (a fluorescent *in vitro* transcriptional

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oscillator) into small, cell-sized droplet microreactors. The oscillator reaction system involves seven DNA strands, two enzymes, two transcribed RNA species and several intermediate species. Using a simple vortexing technique, we generated emulsions of water-in-oil droplets that contained the oscillator, with volumes ranging from  $\geq 16$  pl down to  $\leq 33$  fl. Thousands of individual oscillating droplets were followed simultaneously by optical microscopy, and features such as the period and amplitude of each droplet's fluorescence trace were measured using automated analysis procedures.

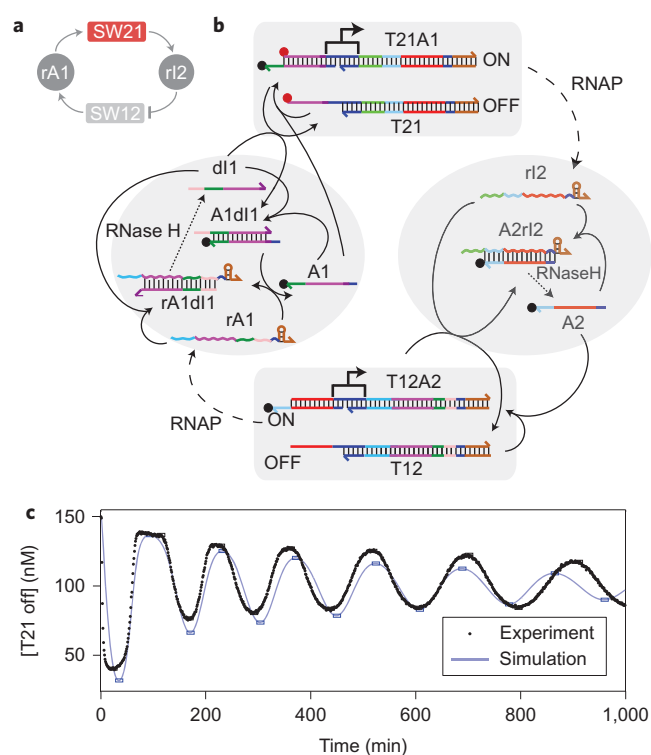
As anticipated for small-number effects, the diversity of behaviours increased in smaller droplets. Surprisingly, however, we found that the diversity could not be explained by the inherent stochasticity of chemical reactions that involve small numbers of molecules, which is often used as an explanation for variability within living cells<sup>33,34</sup>. Instead, our experimental data were more consistent with models in which stochastic partitioning of key low-concentration species (enzymes in particular) introduced diversity in the dynamical behaviour by providing variability in the initial conditions for the oscillator. Thus, the variability observed in our population of oscillators bears more similarities to the 'cell-division noise' caused by the unequal distribution of the molecules of dividing cells between their daughter cells<sup>35,36</sup>.

## Results and discussion

**A synthetic transcriptional oscillator.** Schematic representations of the *in vitro* transcriptional oscillator used in the present study are shown in Fig. 1a,b. Its operation principle and quantitative description are discussed thoroughly by Kim and Winfree<sup>24</sup> and Franco *et al.*<sup>25</sup>. Briefly, it is based on two transcriptional switches, SW21 and SW12 (also termed 'genelets'), that mutually regulate their activity through RNA transcripts rA1 (activator) and rI2 (inhibitor). The genelets are composed of double-stranded DNA templates (T21A1 and T12A2) that contain the promoter sequence of RNA polymerase (RNAP) from bacteriophage T7. The non-coding strand of the genelets is nicked in the promoter region, and removal of the activating DNA strands A1 or A2 by toehold-mediated strand displacement results in strongly reduced transcriptional activity. RNA species rI2, which is transcribed from T21A1, can inhibit transcription from genelet SW12 by displacing A2 from T12A2. By contrast, RNA species rA1, transcribed from T12A2, can activate genelet SW21 by displacing dI1 strands from A1dI1 duplexes; A1 strands are thus released, and can bind to T21. As a result, the two genelets constitute an overall negative feedback loop, which exhibits oscillatory behaviour for appropriate parameter settings. To prevent unlimited growth of RNA concentrations, RNase H is added to degrade, both selectively and processively, RNA that occurs in RNA/DNA hybrid duplexes<sup>37</sup>.

Experimentally, the state of the genelets is read out by fluorescence collected from the dye-labelled T21 strands. A typical fluorescence trace recorded from the transcriptional oscillator system is shown in Fig. 1c. As shown in the figure, experimental data are well reproduced by a theoretical model (the extended model from Kim and Winfree<sup>24</sup>) that captures the most-important chemical reactions (Supplementary Section Modelling). The model contains 17 ordinary differential equations with 24 species and 24 rate parameters. The model parameters were chosen within a realistic range to obtain a least-squares fit to data collected from three distinct operating points of the oscillator, which we named 'sustained' (Fig. 1c), 'damped' and 'strongly damped'.

The enzyme concentrations for the 'sustained' tuning of the oscillator system are [RNase H]  $\approx 5$  nM and [RNAP]  $\approx 200$  nM, and from 80 nM to 500 nM for the oligonucleotide components (Supplementary Section Methods). In droplets with subpicolitre volumes, some of the species are present at only relatively low copy numbers. For instance, in a droplet with radius ( $r$ ) of 2  $\mu\text{m}$



**Figure 1 | An *in vitro* transcriptional oscillator.** **a**, Schematic representation of the two-switch negative-feedback oscillator circuit. Arrowheads indicate activation of a downstream component, and the blunt end indicates inhibition. **b**, Molecular representation of involved reactions. DNA strands are represented by coloured lines. Complementary sub-sequences are coloured identically. RNA signals are represented by wavy lines. Black dashed lines connect transcription substrate and product, and dotted lines connect degradation substrates and remaining products. Switches SW12 (SW21) from **a** consist of genelets T12 (T21) that can be switched to a transcriptional active state by hybridization of an activator strand A2 (A1), which completes the promoter sequence for T7 RNAP (boxes with arrows indicate the direction of transcription). Inhibitor strands dI1 (rI2), which are complementary to activators, switch transcription off by toehold-mediated strand displacement. Excesses of activator A2 and inhibitor dI1 act as thresholds by sequestering free RNA signals up to a certain level, and thus cause a delay in the negative feedback loop that leads to oscillatory behaviour. Genelet T21 is labelled with a fluorophore (red circle) and A1 is labelled with a quencher (black circle), which results in high fluorescence for low transcription activity and vice versa. DNA sequences are given in the Supplementary Methods. **c**, The fluorescent time trace of switch T21 (black) and corresponding fit of the extended model (blue) of the oscillator circuit exhibit sustained oscillations. The eventual decay of oscillator amplitude and period is attributed to the build-up of incomplete RNA degradation products.

(which corresponds to a volume ( $V$ ) of 33 fl) there will be  $\sim 100$  molecules of RNase H, 4,000 RNAPs and the lowest DNA copy numbers will be  $\sim 1,600$ .

**Generating a population of oscillators in microdroplets.** To study the effect of compartmentalization into small volumes on the dynamical behaviour of the transcriptional oscillator, we encapsulated the oscillator components within microemulsion droplets. Water-in-oil droplets were prepared using a non-ionic surfactant<sup>38</sup>, which resulted in microemulsions that were stable for days. A simple vortexing procedure generated a broad droplet-size distribution, with radii that ranged from  $\sim 1$   $\mu\text{m}$  to above 20  $\mu\text{m}$  (Methods). Owing to the non-ionic nature of the surfactant, adsorption of molecules to the droplet boundaries was presumed to be very low. This was validated by control fluorescence measurements on emulsions prepared solely with











