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Synchrony between Genetic Repressilators in Sister Cells in Different Temperatures

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***Abstract*—**We used live *E. coli* containing synthetic genetic oscillators to study how the degree of synchrony between the genetic circuits of sister cells changes with temperature. We found that both the mean and the variability of the degree of synchrony between the fluorescence signals from sister cells are affected by temperature. Also, while most pairs of sister cells were found to be highly synchronous in each condition, the number of asynchronous pairs increased with increasing temperature, which was found to be due to disruptions in the oscillations. Finally we provide evidence that these disruptions tend to affect multiple generations as opposed to individual cells. These findings provide insight in how to design more robust synthetic circuits and in how cell division can affect their dynamics.

***Keywords*—**Repressilator, robustness, synchrony, synthetic biology.

I. Introduction

GENETIC circuits are capable of performing tasks such as time keeping [1], state holding [2], and signal modulation and multiplexing [3]. Naturally occurring circuits responsible for these critical tasks have evolved to be sensitive to specific inputs but robust to external fluctuations such as transient environmental changes [4]-[6]. Such behavior is necessary to regulate periodic cellular processes operating under a wide range of conditions, while maintaining efficiency to respond to environmental signals.

Synthetic versions of some important naturally occurring circuits have been engineered [7], [8]. These synthetic constructs aim to allow the programming of novel biological functions but also aid the understanding of the behavior of naturally occurring circuits, which may allow enhancing their performance as well. To match the performance of the natural circuits, the components of the synthetic circuits must be carefully selected such that both the desired behavior and level of robustness are attained [9]. For this purpose, synthetic circuits utilize chemical components whose physical and chemical properties are well characterized [10].

One example of a synthetic oscillator is the repressilator engineered by [7]. This circuit consists of three genes organized in a ring topology, each inhibiting the expression of a neighboring gene. These interactions form a negative feedback loop, which causes the protein levels of the component genes to oscillate over time. Additionally, one of the component genes is used to control a reporter gene producing green fluorescent proteins, which allows visualizing the system's behavior using fluorescence microscopy. Such oscillator could be used e.g. for time keeping, synchronization via phase-locking, or signal modulation and multiplexing [11].

Temperature is one environmental factor that is known to affect most cellular processes, e.g. by modulating the gene expression dynamics. Evidence suggests that natural time keeping circuits, such as circadian oscillators, have evolved to be robust against temperature fluctuations [4], [5], [12]. In contrast, previous studies of synthetic oscillators have found that the constructed circuits are not immune to temperature changes. In one study, the period of the oscillator was found to decrease monotonically with increasing temperature between 25 and 37°C, causing over two-fold change, presumably because changes in temperature affect the thermodynamics of all the cellular processes [13]. Our previous study on the dynamics of the Elowitz repressilator provides evidence of a similar pattern, but also notes that the most cells exhibit disrupted oscillations for temperatures over 37°C. Evidence was then provided that this is due to loss of functionality of one of the component proteins [14].

In this work, we use live *E. coli* cells containing a synthetic genetic repressilator [7] to study how the synchrony between sisters cells changes as a function of temperature and the resulting changes in the robustness of these circuits. In our cells, the loss of synchrony is inevitable since, following cell division, it is not maintained by any process between the sister cells [7]. This would lead to tangible phenotypic differences between them, provided that the clocks were used to regulate some key cellular process. Here we quantify such degree of asynchrony as a function of temperature. The findings will be important in providing insight in designing more robust synthetic circuits, and in understanding the behavior of naturally occurring circuits.

II. Materials and Methods

A. Cell Culturing and Microscopy

Cells of *E. coli lac*$^-$ strain MC 4100 containing the repressilator and the reporter plasmids were generously provided by M. B. Elowitz, Princeton University, NJ, USA. The cells were grown in minimal media with 2 mM $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich, USA), 7.6 mM $[NH_4]_2SO_4$

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(Sigma Life Science, USA), 30 mM $FeSO_4 \cdot 7H_2O$ (Sigma Life Science, USA), 1 mM EDTA (Sigma Life Science, USA), and 60 mM KH_2PO_4 (Sigma Life Science, USA) (pH 6.8) supplemented with 0.5% glycerol (Sigma Life Science, USA) and 0.1% casaminoacids (Fluka Analytical, USA) overnight at 28, 30, or 37°C with shaking at 300 rpm to an optical density of 0.1 at 600 nm. Next, cells were diluted into fresh media and a few ml of the culture was placed between a cover-slip and a slab of 2% low melting agarose in minimal media. During time lapse microscopy, the temperature of the samples was kept stable by a control chamber (FCS2, Bioptechs, PA, USA). Images were obtained every 15 min for 10 h using a Nikon Eclipse (TE2000-U, Nikon, Tokyo, Japan) inverted C2 confocal laser-scanning system with a 100 Apo TIRF (1.49 NA, oil) objective. GFP fluorescence was measured using a 488 nm laser (Melles-Griot) and a 515/30 nm detection filter. For image acquisition, Nikon EZ-C1 software was used.

B. Image Analysis

The cells were manually segmented in the fluorescence images in each frame of the time series (automatic segmentation is problematic due to the oscillatory signal). Afterwards, cell lineages were established such that a cell (segment) is associated to the cell with largest overlapping cell in the previous frame, after correcting for global translation between the two frames. A cell division was recorded in the case where two cells were associated with the same cell in the previous frame. Finally, the average fluorescence intensity was extracted from each frame and each cell for further analysis.

C. Functionality and Estimation of the Periods

Since a large fraction of the cells do not appear to exhibit oscillations [7], we used the criterion proposed by [7] to categorize the cells as functional or dysfunctional, and only included the functional cells in the further parts of our analysis. In this method, the power spectral density (estimated using discrete Fourier transform) of the signal is compared with that of a decaying exponential with a time constant of 90 min, the measured lifetime of the fluorescent protein [7]. Cells with spectra exhibiting peaks higher than 3.1 times the background spectral density at frequencies of 0.2 to 0.5 h^{-1} were classified as oscillatory. This method was applied to each branch of the lineage trees to determine the functionality of the youngest cells, whereas the other cells were considered to be functional if they had at least one functional child.

The period of oscillations were estimated using the zeros of the autocorrelation sequence of background-corrected intensity signals [14]. In this method, the raw intensity signal is fit with a quadratic polynomial of time, in least-squares sense, to estimate the background trend (caused e.g. by accumulation of GFP and photobleaching). Next, the background trend is subtracted, the residual is scaled to unit power, and the autocorrelation sequence is computed. The period can be estimated by locating the first and third zero of this sequence, as they are expected to occur at lags of 1/4 and 5/4 times the period.

D. Estimating Robustness and True Period Distributions

Particularly at higher temperatures, the period distributions were observed to exhibit bimodality [14]. This might be caused by either the repressilator or the reporter failing, causing an apparent doubling of the period [14]. Higher-order harmonics are not expected to be present due to the finite measurement time.

For this, we find the maximum likelihood estimates using a model of a single normal distribution (given by the mean and standard deviation of the data) and a mixture model of two normal distributions, with the mean and variance of the second equal to twice that of the first (found using an iterative expectation maximization algorithm [15]), using the measured periods in each condition. The appropriate model is selected using a likelihood ratio test with a significance level of 0.01, that is, the bimodal model is only selected if it fits significantly better than the unimodal one. Finally, in the bimodal case, the robustness of the population is determined from the total probability mass in the first normal distribution, and the robustness of the individual cells are determined by a maximum-a-posteriori classifier given the estimated parameters.

III. Results

We analyzed time series of cells with repressilators imaged in three different temperatures: 28, 30, and 37°C. The time series were sampled every 15 min and were 10 h in duration. The image analysis process produced a total of 172, 186, and 683 cells in 28, 30, and 37°C, respectively. Fig. 1 shows examples of confocal microscope images of a few cells and the corresponding extracted intensities.

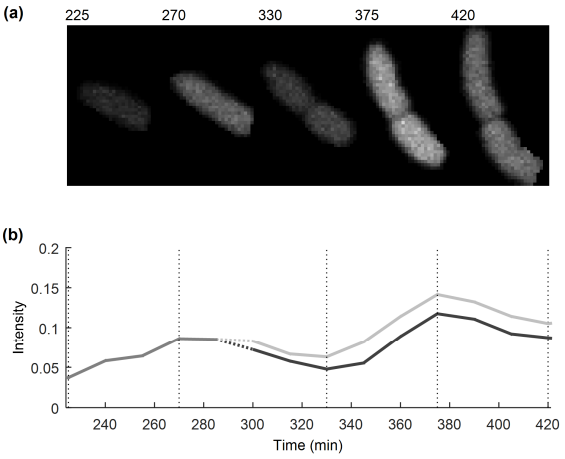

Fig. 1 (a) Example images of related cells with repressilators in five different time points, and (b) the corresponding mean intensities extracted from these cells. The vertical dashed lines indicate the time points corresponding to the images

First, we computed the fraction of functional cells, estimated the period of each functional cell, and finally estimated the distribution of periods and robustness in each condition (see methods). Here functional cells are those that

exhibit oscillations of "strong enough" power, and robust cells are those that exhibit oscillations at the fundamental frequency (and not some harmonic). Summary of the statistics is shown in Table I.

TABLE I
FUNCTIONALITY, PERIOD STATISTICS, AND ESTIMATED PERIOD DISTRIBUTION IN DIFFERENT TEMPERATURES

Statistic	28°C	30°C	37°C
Functionality	0.64	0.83	0.80
Period mean	384	252	245
Period sd	84	93	98
Fit period mean	384	252	150
Fit period sd	84	93	51
Fit robustness	1	1	0.37

Units of time are in minutes. Period mean and standard deviation (sd) were extracted from the data, and estimated period mean, sd, and robustness were obtained by fitting the model (see methods).

The results indicate that the functionality is lower in the 28°C condition (p-value of 1.56×10^{-4} in one-tailed binomial test with null hypothesis of equal distributions), and similar in the 30°C and 37°C conditions (p-value of 0.09). We also found that the mean period decreases with a temperature increase from 28°C to 30°C (p-value of 1.84×10^{-17} in one-tailed Welch's t-tests with null hypothesis of equal means), and is similar in 30°C and 37°C conditions (p-value of 0.28). The coefficients of variation (standard deviation over the mean) extracted from the periods are 0.22, 0.37, and 0.40 for 28, 30, and 37°C, respectively, which suggests that the relative variations in the periods increase with temperature. These results are in agreement with previous findings [14].

Since only around 40% of the cells imaged under 37°C were found to be robust, we further computed the statistics using only the robust cells in this condition. The mean (standard deviation) period of the robust cells is around 150 (51) min, resulting in a coefficient of variation of 0.34. In comparison to the whole population of the cells in the 37°C case, the robust cells have significantly lower mean period compared to the 30°C case (p-value of 2.50×10^{-15}), suggesting that an increase in temperature results in a decrease in the period of oscillations of "properly" operating repressilators throughout the whole region. In addition, the stochasticity in the period duration appears to be similar in the 30 and 37°C conditions and lower in the 28°C condition.

Next, in these data, we located each pair of sister cells in which both of the sister cells were functional. We found 32, 53, and 187 such pairs in 28, 30, and 37°C conditions, respectively, and 35 such pairs in the robust cells of 37°C condition.

We first tested if a robust cell is more (or less) likely to have a robust sister cell than is expected by chance, in the 37°C condition. In our data, we found 112, 40, and 35 pairs where none, one, or both of the cells were robust, respectively, suggesting that the number of pairs where either none or both sisters are robust are overrepresented. More specifically, there is about a 0.64 chance for a robust cell to have a robust sister, and about a 0.85 chance for a non-robust cell to have a non-robust sister (cf. 0.37 in Table I). The significance of this correlation was confirmed by computing the p-value of one-tailed Fisher's exact test with the null hypothesis that being robust or not is independent in the sister cells, resulting in a p-value smaller than 1.29×10^{-10}.

Next, we computed the correlation between the intensity signals of each pair of sister cells. This correlation results from loss of synchrony caused both by division and variations in the behavior of the cells over their lifetime (i.e. variations/drift in the period and noise in the intensity signal). The distributions of correlation coefficient extracted from each pair of cells are shown in Fig. 2, and the mean and standard deviation of the coefficients is shown in Table II.

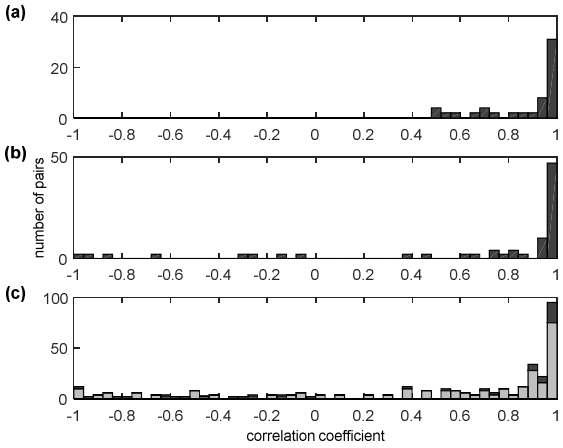

Fig. 2 Distributions of correlation coefficients between functional sister cells in (a) 28, (b) 30, and (c) 37 °C. In the 37 °C condition, the pairs where both cells are robust are represented in dark gray bars, while the others are represented in light gray

TABLE II
CORRELATION BETWEEN SISTER CELLS IN VARIOUS TEMPERATURES

Statistic	28°C	30°C	37°C	37°C, robust
Correlation mean	0.87	0.66	0.42	0.63
Correlation sd	0.16	0.58	0.66	0.58

We found that as the temperature increases, the sister cells lose correlation, on average (p-values of 3.17×10^{-3} and 9.90×10^{-4} for 28 vs. 30°C and 30 vs. 37°C in one-tailed Welch's t-test with the null hypothesis that the means are equal). This loss of correlation could be due to the increase in the noise of the period as a function of the temperature, which appears to follow a similar pattern. Accordingly, since the non-robust cells contribute much of the variation in the 37°C condition, the correlation is restored to a level comparable to the 30°C condition when only the robust cells are considered. Interestingly, Fig. 2 reveals that in each condition, most of the cells are very highly correlated. However, increases in the temperature results in pairs of cells with wider range of correlation coefficients, including a sizable number of pairs whose series are strongly anticorrelated.

IV. Conclusion

We used live *E. coli* cells containing genetic Repressilators to study how the synchrony between synthetic genetic repressilators contained in sister cells changes as a function of both the temperature and the affected robustness of the cells.

We found that the temperature affects both the mean and the variability of the synchrony between sister cells, as measured by the correlation coefficient between their intensity time series. While in each condition most pairs of sister cells are highly correlated, the number of uncorrelated and anticorrelated pairs grows with increasing temperature. These values result in an apparent reduction in the synchrony between the sister cells for the population as a whole.

However, in the 37°C condition, the non-robust pairs (i.e. cells whose oscillations become disrupted) were found to be responsible for these unlikely pairs, and the synchrony of the cells that remain robust is comparable to the 30°C case, as predicted by the changes in the stochasticity of the period. Finally, we found that a robust/non-robust cell is more likely to have a sister with similar than the opposite behavior, suggesting that the disruptions in the oscillators propagate to successive generations.

To explain these results, we provide evidence that the changes in the synchrony between the sister cells are reflected with changes in the stochasticity of the period of oscillations. Such stochasticity is expected to result in the sister cells randomly drifting to different behaviors over time. This hypothesis would explain the changes in synchrony both as a function of changes in the temperature and as a function of the changes in the robustness of the cells. However, we note that it remains unclear if other noise sources, such as the stochasticity of partitioning the repressilator or GFP plasmids, differ in the different conditions, and if they have a significant effect on the synchrony of cells of common ancestry.

These results further support the hypothesis that in higher temperatures the repressilators become disrupted, which might be due to the component protein CI losing functionality in these temperatures [14]. Furthermore, we have provided evidence that such failure would not only cause a disruption in the oscillatory signals, but also the loss of synchronization between similar the clock signals, which might be important if independent clocks are used to drive downstream circuits.

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