Wavelet Analysis Reveals Period Wandering in Circadian Neurons

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Abstract

Period variability in circadian oscillators is a consequence of the stochastic nature of the underlying chemical processes. This variability is significant when considering the interaction of circadian neurons in the suprachiasmatic nucleus (SCN) and their response to external signals. We use the Morlet continuous wavelet transform to measure the period of the noisy nonstationary circadian oscillator, and demonstrate its use on data from both biological experiment and a stochastic model. Wavelet analysis of the period of individual dispersed cells shows that they sometimes cycle at longer than circadian periods, while cells in a SCN tissue slice do not exhibit these longer periods. We demonstrate that the distinct period variations observed in experiments can be reproduced in a stochastic model by an adjustment of parameter values guided by bifurcation analysis of its nonlinear dynamics. The resulting model suggests that the long periods of individual cells could be produced by switching between the period determined by the PER-CRY feedback loop or the CLOCK-BMAL1 feedback loop of the gene regulatory network. The period of individual cells are then stabilized in SCN tissue with the PER-CRY feedback loop determining the period.
1 Introduction

In mammals the suprachiasmatic nucleus (SCN), a region of about 20,000 neurons in the brain is the master circadian clock (32). Circadian rhythms in gene expression occur in individual neurons, however the period precision of the master clock is shown to degrade in experiments which sever intercellular communication. Herzog et al. (20) measured the period distribution and cycle-to-cycle variability of dispersed neurons and of SCN explants, observing a narrower period distribution in explants than in dispersed neurons. Aton et al. showed that vasoactive intestinal polypeptide (\textit{Vip}^{-/-}) and its receptor (\textit{Vipr2}^{-/-}) knock-out mice exhibit significantly broader period distributions in both behavioral and SCN firing rate periods (3). The precision of the SCN master clock helps coordinate the timing of slave peripheral oscillators which have been found in the liver, lung, and skeletal muscle (32),(38). These peripheral oscillators may require a precise reference clock such as the SCN to orchestrate ultradian rhythms and phase relationships in local physiology and behavior. The source of precision in the SCN rhythm is unknown, but is thought to be due to cell-cell interactions within the SCN (20). Understanding how the noisy rhythm of gene expression in individual cells produces a precise rhythm at the tissue level is an important challenge for systems biology modeling.

Bernard et al. (7), To et al. (35), and Vasalou et al. (36) modeled synchronization in a heterogeneous population of individual cells, where each cell is a deterministic oscillator at a different fixed period. Forger and Peskin (16) and Gonze and Goldbeter (19) measured the period distribution of a single cell modeled as a 5-state or 3-state stochastic system respectively. The variance of the single-mode period distribution produced by these models increased in proportion to $n^{-rac{1}{2}}$ as the molecular population was decreased or the bifurcation point was approached. In a linear system, the variance of the period distribution due to stochastic noise increases in proportion to $n^{-rac{1}{2}}$ where $n$ is the molecular population count. In a nonlinear system, the change in period with molecular count can be less predictable. For nonlinear systems operating near a point of chemical instability, stochastic fluctuations become significant and a deterministic model is inadequate (17),(18). Stochastic models provide a powerful opportunity for testing potential mechanisms in the SCN for achieving precise rhythms.

Song et al. (34) demonstrated that a simple model consisting of interlocked positive and negative CREB feedback loops could behave as both a bistable switch and an oscillator depending on the relative strengths of the two feedback loops. They used bifurcation analysis to examine the nonlinear
dynamics of the model and found a bimodal distribution of CREB in the bi-stability region of the dual feedback loops. They also discussed the interaction of stochastic noise with the bistable switch and oscillatory functions of the system. Leloup and Goldbeter (24) found birhythmicity (coexistence of two stable limit cycle oscillations) in a deterministic model of the Drosophila circadian gene regulator network. Brandman and Meyer provide a review of the function of multiple feedback loops in biological signaling systems.(10)

In this study we measure the period precision and persistence of individual cells, using the continuous wavelet transform (CWT), to better understand the interaction between cell and SCN system robustness. We develop a stochastic version of the Leloup and Goldbeter gene regulatory network model, and find that stochastic noise due to low molecular population is not enough to produce the period variations observed in the dispersed cells. We use bifurcation analysis and numerical simulation of the model to identify a potential mechanism for the period switching. The period switching of the circadian oscillator with variation in the activation of $Bmal1$ transcription indicates that switching between the two primary feedback loops of the gene regulatory network may be an explanation for period instability in cells and period precision in the SCN due to synchronization of the cells.

Our wavelet analysis of the biological data shows that the period distribution is not Gaussian with a simple increase in variance as the molecular count is decreased. Our larger 16-state stochastic model, in contrast to smaller 3-state and 5-state models (19),(16), is able to produce a non-Gaussian period distribution that changes shape as the molecular count is decreased. It has been hypothesized before (27) that the instability of individual cell oscillations might be due to their proximity to a fixed point bifurcation where oscillations cease, and that inter-cellular communication is capable of restoring rhythms. To et al. (35) used proximity to a fixed point bifurcation to produce a mixed population of oscillating and non-oscillating cells. Our bifurcation analysis and numerical simulation results show that the shape of the period distribution depends on which bifurcation point you choose. We show that the source of instability in individual cell oscillations could be switching, driven by stochastic noise, between 2 feedback loops in the gene regulatory network. The stability of individual cell oscillations is an important property affecting SCN system behavior when cells are communicating. Finally, the method of analysis we have developed: wavelet analysis of non-stationary oscillations and bifurcation analysis of a deterministic model (a stochastic model in the limit of a large number of molecules) used to guide stochastic numerical experiments, is of general use for stochastic model discrimination.
Figure 1: Comparison of wavelength (equivalently: period) evaluations for a non-stationary oscillator. (a) - A cosine with period 24 interrupted by a pause of length 60, with best fit by FFT-NLLS superimposed. (b) - A wavelet ridge plot displaying time-frequency estimates for period at each time-point; a dark point indicates the dominant frequency (y-axis) at each given time-point (x-axis). FFT-NLLS suggest 3 periodic components are present, with periods 40.8, 25.5, and 22.67. The wavelet method, by comparison, accurately indicates that the signal has a strong periodic component at a period of 24 that is only present intermittently.
Period variability resists analysis by traditional methods such as Fourier transforms, $\chi^2$ periodogram, and autocorrelation analysis (see Figure 1, for example). These methods assume that the signal being analyzed is strongly stationary, with a fixed and unchanging period and/or autocovariance function. Biological systems do not meet these criteria, exhibiting “sloppy” behavior (20). As such, they are poorly suited to analysis by these methods. An alternative class of methods known as time-frequency analysis (9) – including wavelets, windowed Fourier transforms, and Wigner-Ville distributions, among others (29) – permit trade-offs to be made between the precision of analysis over time and estimation of frequency components. This allows for the examination of signals with periodic components that change over time, and characterization of not only what frequencies are present, but when they enter and exit the data (28), (14),(12). Even when multiple non-stationary oscillators are superimposed, time-frequency analysis can recover frequency modulation laws that describe each component (33).

For analysis of the data presented in this paper, we selected the Morlet wavelet, which is closely related to the familiar tools of Fourier analysis (29), (6), and can be considered as a technique to adaptively window a Fourier transform in such a way that the size of the window is adjusted to each frequency being analyzed. Wavelets, including the Morlet wavelet, have been successfully used to analyze circadian rhythms (31), (4), as well as other biological data across a wide range of time scales such as ECG signals (2), (23), (30), EEG signals (6), human blood-flow dynamics (8), and yeast RNA transcription data (22). We extend the usual techniques used to characterize the behavior of single realizations of the data by introducing a methodology for visualization and comparison of distributional properties when the periodic behavior of the oscillators is stochastic.

The remainder of the paper is organized as follows. In Section 2, we describe the biological experiments used to collect both uncoupled and coupled cell data, review methods for estimating the period of an oscillatory signal, and describe the computational models. In Sections 3.1 and 3.2, we present two novel methods for combining and presenting CWT data as well as the results of the wavelet analysis of the experimental data. In Section 3.3 we demonstrate the use of bifurcation analysis on a deterministic model to determine a set of parameters for which the corresponding stochastic model reproduces the major features of the distributional information of the data. In Section 4 we discuss the biological implications of the results and outline our future work.
2 Methods

2.1 Experimental Procedure for Collecting Circadian SCN Data

The dispersed cultures of SCN cells that provided the bioluminescence data used in this paper were generated as described in Herzog (37). Briefly, SCN cells were harvested from mice genetically modified to express a fusion mouse Period2-firefly luciferase protein. The cells were dispersed in culture medium containing luciferin. Light was emitted when and where the PER2 protein was made.

Single-cell bioluminescence measurements began on the 4th day following harvesting, and were continued for at least 6 days. Data were recorded using a Versarray 1024 cooled-CCD camera (Princeton Instruments). Photon measurements for each individual neuron were binned over 1 hour using WinView software, and quantified using NIH Image. Each number in the data analyzed is proportional to the total number of photons emitted by each cell over the course of an hour.

Recordings from single cells clearly exhibited periodic behavior, but also included moving baselines, high-frequency noise, and amplitudes and fundamental frequencies that change over time, rendering them resistant to analysis via procedures that assume strongly stationary data. In order to accurately measure the phase, period and amplitude of the bioluminescence oscillations, we utilized the Morlet wavelet transform, which is capable of identifying periodicities in data in which these parameters are constantly changing.

2.2 Wavelet Tools for Analysis of Circadian Data

The Morlet wavelet has seen limited use in analysis of circadian time series (31)(4). The direct application of the Morlet wavelet allows for simultaneous estimation of phase, frequency, and amplitude of a particular data set while simultaneously detrending it, all without the strong parametric assumptions that cause difficulty for traditional methods (4).

Briefly, applying the Morlet wavelet to a data series generates a Continuous Wavelet Transform table (CWT table): a complex-valued field across scales (corresponding roughly to frequencies), and translations (corresponding roughly to time). The magnitude of the complex value at any scale and translation indicates the approximate strength of the frequency corresponding to the selected scale at a time corresponding to the translation (29). By selecting a series of points across translations at which the magnitude of
Figure 2: Three example datasets for PER2:LUC bioluminescence data, illustrating features that can make such data difficult to analyze: (a) increasing amplitude and period over time, (b) high frequency noise, and (c) a decreasing baseline.
the CWT reaches local maxima (the “CWT ridges”), estimates of the frequency evolution of the periodic components of the signal can be obtained. In some cases, particularly when noise levels are high and multiple oscillators may be contributing to the signal, simply selecting the global or local maxima at each translation may not be the optimal method of extracting the CWT ridge (see (1) for an overview of techniques and their associated issues, and (12) for the “Crazy climber” algorithm used in (4)), however when a single oscillator can safely be assumed to be present, simply selecting the translation-by-translation maximum (11) provides a robust and deterministic method method for obtaining the frequency evolution of the oscillator.

This analysis is quite effective for deterministic oscillators or stochastic oscillators that are approaching the deterministic limit, however for oscillators that exhibit strongly stochastic behavior, distributional properties become much more relevant than individual realizations. We extend the usual method of extracting information on the frequency of oscillations for a particular cell to allow examination and comparison of the distributional behavior of populations of cells, as discussed in Section 3.

2.3 Computational Model

A deterministic model of the mammalian circadian gene network, due to Leloup and Goldbeter (25) and (26) with parameter set 1, was chosen for conversion to a stochastic model because it contained enough detail to trace period instabilities to the underlying biological mechanisms. The model provided PER protein levels for comparison with PER2:LUC biological experiment data, and control of the Per gene transcription rate which was used in modeling the SCN. SCN tissue was modeled by coupling individual cell models into a 2 dimensional 8x8 grid connected through activation of Per transcription by vasoactive intestinal polypeptide (Vip) (35). The topology used for this coupled model was all-to-all connectivity, weighted by the inverse of the distance between cells.

We converted the deterministic model of the mammalian circadian gene network (25) into a stochastic model by changing reaction rates to propensities for discrete reaction events, and converting concentrations of chemical species to population counts. This allowed us to compare the potential contributions of time variation in specific molecular events to the circadian precision in Period gene expression of SCN neurons. Bagheri et al. (5) gives the propensities for the 53 reactions in the cell model, and the conversion from concentration to population count. Parameter values that were
changed during this investigation are noted in the results section of this paper in Table 1. The Stochastic Simulation Algorithm due to Gillespie (17), was used to compute time-dependent solutions. Simulations of individual uncoupled cells were run simultaneously by turning off the coupling. Initial conditions were selected randomly from a previous simulation, which was run long enough for any transients to settle. This randomization of initial conditions along with the random firing of reactions during the simulation gave each cell a unique time history.

3 Results

3.1 Analysis of Period Distribution Properties

As the signals we are considering are stochastic in nature, examining the time-frequency evolution of a single realization of a signal, as proposed in (31) and used in (4) is not sufficient to analyze its distributional properties. In order to simultaneously compare multiple signals, we present two novel methods for combining and presenting CWT data to allow distributional comparison in an explicitly stochastic setting: period distribution plots, and period-duration plots.

Period distribution plots identify the duration for which each cell was oscillating most strongly at the identified period (extracted from the wavelet frequency heat map; Figure 3c,d). A simple histogram is created for each cell (as in Figure 4a), and a population of histograms are assembled into a surface to visualize the distribution of periodicities over a population of cells (Figure 3e,f). This quantifies the dominant period as well as its variability over time, both for each individual cell and across the population of cells.

As period histograms suppress temporal information about the frequency distribution, particularly information about which frequencies persist and which frequencies are transient, we introduce period-duration plots in order to capture this information over a population of cells. Each dominant oscillation is selected from the signal, and its duration and average period calculated. More persistent ridges appear further towards the right-hand side of the plot, and longer frequencies appear closer to the top of the plot. Figure 4 displays the relationship between the period histogram for a single cell and the period-duration plot, while Figure 3g,h displays period-duration plots for observed biological signals and simulated signals, respectively. The simulation results are of the Long-Period Stochastic model described in Section 2.3.

These techniques allow us to visualize the behavior of populations of
Figure 3: Period variability plots of PER2:LUC bioluminescence in dispersed SCN neurons (left) and Per mRNA in the stochastic long-period cell model (right): (a) single neuron (b) single stochastic cell model (c-d) wavelet frequency heat map of same single neuron and stochastic cell model (e-f) period distributions of each neuron and stochastically modeled cell over 150 hours (g-h) period duration of all neurons and stochastically modeled cells (+ denotes single cell plotted in a-d).
cells, thus extracting qualitative distributional information from multiple observations, and matching the stochastic behavior of the model to the observed variation in the cells.

3.2 Wavelet Analysis Reveals Two Dominant Periods

CWT coefficients of dispersed cells (Figure 3e) show the random nature of the dominant period. In most cases, the strongest single period is in the 24 to 30 hours range, however the population of cells displays occasional excursions to significantly longer periods. Though there is likely some heterogeneity between individual cells, the ridge distribution plots clearly indicate that there are not two distinct groups of cells oscillating with 24-30 hour periods and circa 55 hour period respectively, but rather that individual cells change period during the time course of the experiment. Ridge plots of the individual cells show continuous ridges until a "switch" occurs between the circadian and longer period. A frequency-duration plot (Figure 3g) shows that the 55 hour periods last for up to 4.5 days.

Figure 5(a,c) shows the period distribution of coupled cells in a SCN slice. It can be seen from the ridge distribution plot that the circadian mode becomes more precise, an expected consequence of synchronization. In the period duration plot, the maximum persistence of the 55 hour mode has been reduced to less than 3.3 days.
Figure 5: Ridge distribution and period duration plots of (a,c) SCN tissue PER2:LUC bioluminescence and (b,d) stochastic model of coupled cells, model coupling strength = 0.4, $K_{IB} = 6.0$, $\nu_{SP} = 1.1$, and $\nu_{SB} = 0.9$. 
The presented visualizations allow for examination of novel behavior in SCN cells – the presence of two distinct periods of oscillation, both potentially present within any particular SCN cell – as well as clear visualization of intracellular period variability as previous observed in (20). The persistence of the longer period of SCN cells is substantially reduced in the presence of coupling, and the intracellular period variability is visibly reduced.

### 3.3 Modeling Provides a Possible Mechanism

We considered three models: our original model (Stochastic Leloup and Goldbeter), a version of the original model with the $Per$ transcription rate near its non-oscillatory bifurcation point (Stochastic To), and a new Stochastic Long-Period model that better reproduces the data. The model parameters are given in Table 1 below. The Stochastic Leloup and Goldbeter model was unable to reproduce the period instability as described above. Comparison of model simulations with measurements of dispersed cells shows (Figure 6a) that at populations able to reproduce the period and synchronization observed in the deterministic model, the period distribution is narrower than that of the biological data. As the molecular population is lowered the period distribution widens (Figure 6b), but the ability of the system to synchronize is lost. This is due to the loss of coherent oscillation (and amplitude) of the individual cells.

<table>
<thead>
<tr>
<th>Model Name</th>
<th>$\Omega$</th>
<th>$\nu_{SP}$</th>
<th>$K_{IB}$</th>
<th>$CS$</th>
</tr>
</thead>
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<tr>
<td>Stochastic Leloup and Goldbeter (25)(26)</td>
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<td>1.5</td>
<td>2.2</td>
<td>NA</td>
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<tr>
<td>Stochastic To (35)</td>
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<td>1.1</td>
<td>2.2</td>
<td>1.1</td>
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<tr>
<td>Stochastic Long-Period</td>
<td>50</td>
<td>1.1</td>
<td>6.0</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 1: Model parameter values changed from Leloup and Goldbeter (25)(26) with parameter set 1, or To et al. (35): cell volume $\Omega$, $Per$ transcription rate $\nu_{SP}$, $Bmal1$ inhibition constant $K_{IB}$, and coupling strength $CS$.

The Stochastic To model widens the period distribution (Figure 7a). However, the frequency-duration plot of the uncoupled cells (Figure 7b) shows a circadian mode, but no second mode at the longer 55 hour period. In this case, the system retains its ability to synchronize. The frequency-duration plot of the coupled cells (Figure 7d) shows narrowing of the period distribution, as in SCN tissue.

Knowledge of how the period of the deterministic Leloup and Goldbeter model, from (25) and with parameter set 1 from (26), depends on the pa-
Figure 6: Ridge distribution plots as the molecular count is lowered from (a) $\Omega = 50$ to (b) $\Omega = 25$ in a stochastic version of the Leloup and Goldbeter model(25).

Figure 7: Ridge distribution and period duration plots for the Stochastic To model of (a-b) uncoupled and (c-d) coupled cells near the $Per$ transcription $\nu_{SP} = 1.1$ bifurcation ($\Omega = 50$, coupling strength = 1.1).
Parameter values was used to develop a Stochastic Long-Period model that is better able to reproduce a desired period distribution. In order to determine how the nonlinear dynamics of the gene regulatory network could produce the longer 55 hour period, dynamic analysis of the deterministic model was employed to determine the dependence of the period on the model parameters. Figure 8 illustrates the main feedback loops of this model and shows (thicker arrows) the pathways associated with parameters that have values that can produce long periods comparable to the data.

Figure 8: A model of the mammalian circadian gene regulation network (26) which includes three key genes, Per, Cry, and Bmal1, and one constitutively expressed gene, Clock. The messenger RNA’s move from the nucleus to the cytosol where they trigger the production of PER, CRY, and BMAL1 proteins. PER and CRY form a heterodimer which re-enters the nucleus along with BMAL1 and CLOCK proteins. BMAL1 and CLOCK form another heterodimer in the nucleus which activates Per and Cry transcription and suppresses Bmal1 transcription. BMAL1-CLOCK and PER-CRY heterodimers form a large inactive complex reducing the BMAL1-CLOCK available for transcription feedback. The thick lines denote the processes which have parameter values that can produce long periods (25) and using parameter set 1 from (26).

XPPAUTO (15) was utilized to compute the period as a function of model parameters (Figures 9 and 10) following the procedure for continu-
ation analysis of an oscillatory system. Leloup and Goldbeter (26) showed that their parameter set 1 requires the presence of both Per-Cry and Bmal1 feedback loops for oscillations to occur. Our analysis identified two sets of parameters that are capable of producing the longer periods observed in the dispersed cell data. The first set (Figure 9) is associated with the PER-CRY feedback loop and requires an order of magnitude change in value to produce the period range. The second set (Figure 10) is associated with the CLOCK-BMAL1 feedback loop or global scale factors, and produces the observed period range with less than an order of magnitude change.

![Figure 9: PER-CRY feedback loop parameters that produce a long period and have one stable oscillatory branch control the production of PER-CRY protein complex and its binding to the CLOCK-BMAL1 protein complex. (a) PER-CRY transport rate from cytoplasm to nucleus (b) PER-CRY to CLOCK-BMAL1 protein complex binding rate (c) PER protein production rate (d) CRY protein production rate (e) PER protein phosphorylation rate.](image)

For the second set of parameters, the period versus parameter functions all have two oscillatory branches connected by an unstable oscillatory segment. Leloup and Goldbeter (26) identified each of the two oscillatory branches with one of the positive or negative feedback loops present in the circadian clock. Song et al. (34) demonstrated that a simple model consisting of interlocked positive and negative feedback loops could behave as both a bistable switch and an oscillator depending on the relative strengths
Figure 10: CLOCK-BMAL1 feedback loop parameters or global scale factors that produce a long period and have two stable oscillatory branches (solid line) connected by an unstable oscillatory range (dashed line), control production of Bmal1 mRNA. Global parameters $\nu_{\text{tot}}$ and $k_{\text{tot}}$ scale transcription and protein production rates of all three key genes. Changes in these global parameters are reflected in the Bmal1 mRNA transcription rate and BMAL1 protein production rate, and so produce the same 2 branch bifurcation behavior found in parameters affecting the production of Bmal1 mRNA. (a) Per gene dissociation constant (b) Bmal1 mRNA transcription rate (c) Bmal1 mRNA degradation rate (d) transcription rate scale factor for all three genes (e) protein production rate scale factor for all three proteins.
of the two feedback loops. Selecting parameter values that allow switching between the oscillatory branches provides a mechanism for producing the bimodal period distribution observed in measurements of the biological system.

The Bmal1 inhibition constant $K_{IB}$ was chosen from the second group of parameters for its maximum period. Stochastic simulation results (not shown) reveal that as its value increases from the lower to the upper oscillatory branch the period distribution widens, but stays limited within a band of 24-48 hours. At the same time, the mean of the period distribution increases from 24 to 48 hours. Combining a value of $K_{IB} = 6.5$ on the upper branch with the value of $\nu_{SP} = 1.1$ near its non-oscillatory boundary produces a cell model (Figure 3) that retains oscillation when uncoupled and produces the bimodal period distribution observed in the biological data.

4 Conclusions

The period distribution of the cell model is an important property for modeling synchronization in a system of coupled oscillators because it has an effect on the formation of clusters of synchronized cells (21). We have shown that new techniques for visualization of wavelet data enable the comparison of measurements of PER2:LUC in individual cells with nonlinear stochastic models. The wavelet visualization revealed that less stable dispersed cells can have periods as long as 55 hours for several cycles before switching to periods near 24 hours, while more stable cells in tissue have periods consistently closer to 24 hours. We found that characteristics of the period variability, its frequency distribution and stability, could be used to distinguish between stochastic model variants, when deterministic versions of these models would produce indistinguishable periods.

We showed that bifurcation analysis of the deterministic model could be used to refine a derived stochastic model to produce the period distribution observed in dispersed cell culture. We reproduced the period distribution in the stochastic model by operating near the bifurcation point in the Bmal1 activation parameter between two stable oscillatory branches. Leloup and Goldbeter (26) identified each of the two oscillatory branches with either the positive or negative feedback loop in the circadian gene regulatory network. Selecting parameter values that allow switching between the oscillatory branches provides a mechanism for producing the observed period distribution. This requires only a modest change in parameter values.

While the single cell Stochastic Long-Period model was shown to syn-
chronize and produce a more precise 24 hour clock, many questions remain to be answered about the coupled system. What intercellular mechanism might lower the Bmal1 transcription rate in the coupled system? Are there other mechanisms that provide the same stability in the coupled system? Finally, how do stochastic noise and connectivity between cells contribute to stability and synchronization observed in the SCN? It may be that the coupled system achieves greater stability while not completely eliminating the longer period through reduced signal-to-noise provided by the inter-cellular connectivity.

Period variations imply that the phase response is also affected (13)(19), so these findings may also be significant for modeling perturbation effects on the coupled system such as light entrainment. In addition to measuring the period, wavelets can be used to extract phase information. Our future work will focus on developing the coupled cell model using wavelet analysis to examine the phase of individual cells as they synchronize and respond to perturbations.

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