YALE JOURNAL OF BIOLOGY AND MEDICINE 92 (2019), pp.169-178.

ORIGINAL CONTRIBUTION

YALE JOURNAL OF BIOLOGY AND MEDICINE
YJBM
CELEBRATING 90 YEARS 1928–2018

What is Phase in Cellular Clocks?

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Four inter-related measures of phase are described to study the phase synchronization of cellular oscillators, and computation of these measures is described and illustrated on single cell fluorescence data from the model filamentous fungus, *Neurospora crassa*. One of these four measures is the phase shift ϕ in a sinusoid of the form $x(t) = A(\cos(\omega t + \phi)$, where t is time. The other measures arise by creating a replica of the periodic process x(t) called the Hilbert transform $\tilde{x}(t)$, which is 90 degrees out of phase with the original process x(t). The second phase measure is the phase angle $F^H(t)$ between the replica $\tilde{x}(t)$ and x(t), taking values between $-\pi$ and π. At extreme values the Hilbert Phase is discontinuous, and a continuous form $F^C(t)$ of the Hilbert Phase is used, measuring time on the nonnegative real axis (t). The continuous Hilbert Phase $F^C(t)$ is used to define the phase $M^C(t_1,t_0)$ for an experiment beginning at time t_0 and ending at time t_1. In that phase differences at time t_0 are often of ancillary interest, the Hilbert Phase $F^C(t_0)$ is subtracted from $F^C(t_1)$. This difference is divided by 2π to obtain the phase $M^C(t_1,t_0)$ in cycles. Both the Hilbert Phase $F^C(t)$ and the phase $M^C(t_1,t_0)$ are functions of time and useful in studying when oscillators phase-synchronize in time in signal processing and circadian rhythms in particular. The phase of cellular clocks is fundamentally different from circadian clocks at the macroscopic scale because there is an hourly cycle superimposed on the circadian cycle.

INTRODUCTION

Recently single cells have been shown to have circadian oscillators using fluorescent markers in clock-related genes [1]. These kinds of fluorescent and luminescent measurements on single cells have been made in a variety of clock systems recently [2-6]. There are three basic properties of oscillators in single cells: the amplitude, period, and phase of each cell's oscillations. The most elusive of these quantities is the phase. How the phase behaves can provide information on how circadian oscillators synchronize in whole tissues or organisms [7]. Oscillators in single cells each appear to have substantial variation in phase between cells [1], but at the macroscopic level of 10^7 cells the ensemble of circadian oscillators appear synchronized when assayed in liquid culture

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Keywords: circadian rhythms, single cell measurements, phase, Hilbert Phase, synchronization, *Neurospora crassa*

Author Contributions: L.M, H-B.S., and JA designed research. Z. D. collected data. H-B.S. developed methods. C.C developed models. C.C, J. H-C., X. Q., E. K. Z. D., and J.A. analyzed data. J.A. wrote the manuscript. We acknowledge the support of the UGA Franklin/OVPR seed grant program, UGA College of Agricultural and Environmental Sciences, the ARCS foundation for support of E. Krach, NSF ECCS-1150042 from NSF Electrical, Communications and Cyber Systems, and NSF MCB-1713746 from NSF Systems and Synthetic Biology (SSB) and NSF Physics of Living Systems (PoLS).

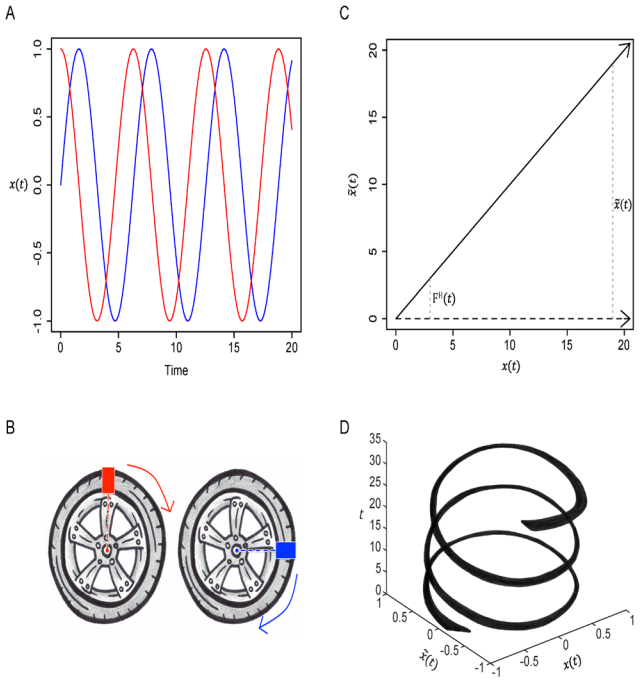

Figure 1. Description of Hilbert Phase. (A) The original process x(t) in red is replicated by a Hilbert transform to $\tilde{x}(t)$ in blue. (B) Creating the replica is analogous to putting two marks on two different tires, which are at 12:00 and 3:00 o'clock to start. (C) The angle $F^{c}(t)$ between the two marks is the Hilbert Phase. One mark corresponds to the original process x(t) in red. The second replica mark corresponds to the Hilbert transform $\tilde{x}(t)$ in blue. (D) The pair (x(t), $\tilde{x}(t)$) defines a number in the complex plane, and as the tires rotate, the pair form a spiral over the complex plane known as the analytic signal.

or in race tubes, for example [8]. How does this phase synchronization arise? In order to address this question there is a need for a clear notion of phase and how to calculate the phase of a cellular oscillator [9]. Whether or not phase synchronization happens may have profound consequences for our health and successful aging [10].

One particular notion of phase relied on heavily in this work is the Hilbert Phase, which was originally developed for signal processing applications [11]. It has come to the fore recently in the analysis of single cell data in *Mus musculus* [12] and *N. crassa* [1]. There has also been increasing interest in its use for identifying phase response curves describing synchronization to light and other entrainment signals [13,14].

Here we develop four inter-related notions of phase of a cellular oscillator, the phase shift, the Hilbert Phase, the continuous Hilbert Phase, and phase in cycles. We use single cell oscillators in the model system, *N. crassa*, to illustrate each of these four interrelated measures of phase and their function in describing "phase." We use these phase measures to examine: (1) phase variation in single cells; (2) the effect of the social environment of cellular oscillators on phase; (3) phase as a function of time as when there is synchronization of oscillators. Each of these topics illustrate how all four notions of phase are measured in concert to provide insights into cellular oscillators.

MEASURES OF PHASE

A fluorescent or luminescent measurement x(t) at each time point t is made on the fluorescence or luminescence of a clock-related gene in single cells. Typically these measurements are taken every half hour over ten, 24 hour days, in the model system, *N. crassa*, used to illustrate the phase calculations [1]. The simplest model for these measurements is a sinusoid of the form $x(t) = A\cos(\omega t + \phi)$, where A is the amplitude of the signal, ω, the frequency of the oscillation, and ϕ, the phase shift of the process. This is sometimes referred to as the hidden periodicity model [15]. If the phase ϕ were constant over time, then the phase shift would capture all of the phase information about each cell and could be extrapolated safely to later times after the zero time point to examine phase relations between cells. The challenge is when the phase shift is not constant in time, as when cells synchronize their phases in time.

Let us suppose we could create a replica $\tilde{x}(t)$ of the

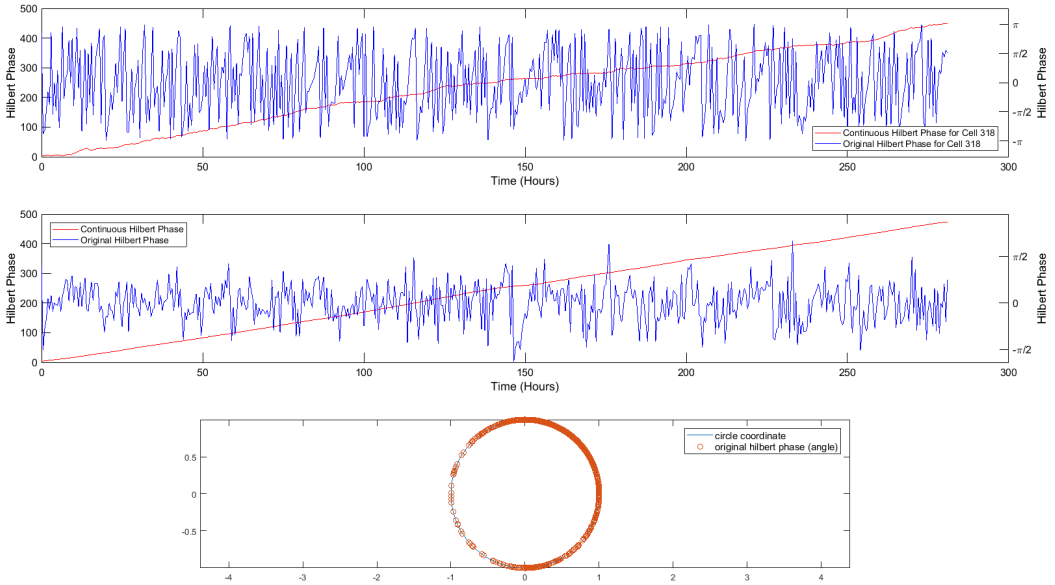

Figure 2. The continuous Hilbert Phase $F^C(t)$ as a trajectory for a single cell (top panel) or averaged over all 1,591 cells is a smooth function of time (in red) while the original Hilbert Phase for cell number 318 (in blue) or averaged over all cells is ragged (in blue) and as an angle is confined to the interval -π to π on the upper panel (see bottom panel). For the top and middle panels the values of the continuous Hilbert Phase $F^C(t)$ are plotted on the left vertical axis; the values of the original uncontinuized Hilbert Phase $F^H(t)$ are plotted on the right vertical axis. On the lower panel is a plot of the original Hilbert Phase $F^H(t)$ as a phase angle from -π to π on the unit circle. Only Hilbert Phase values at the final time point are displayed.

original process x(t) that was 90 degrees out of phase with the original process. For example, if $x(t)$ *were* cos (ωt), then the replica $\tilde{x}(t) = \sin(\omega t)$ would be 90 degrees out of phase (Figure 1A). We can think of each process as a mark on a spinning tire on a car; one tire is marked, and another tire is marked with its mark at 90 degrees from the other mark at time t = 0 (Figure 1B). The two marks, x(t) and $\tilde{x}(t)$ are followed over time. Then we could observe how the original process and the replica change position with respect to each other in time. This is usually done by way of an angular measurement between the marks on each tire. In other words, we can watch the two marks on two tires change position (*i.e.*, angle) with respect to each other in time. The surprise is that under very general conditions (described in Materials and Methods) such a replica $\tilde{x}(t)$ can be created and is called the Hilbert Transform of x(t) [11].

The Hilbert Phase $F^H(t)$ is then defined as the phase angle between the original process x(t) and the replica $\tilde{x}(t)$ (Figure 1C) [16]:

$$F^H(t) = tan^{-1}\frac{\tilde{x}(t)}{x(t)} \quad .$$

This phase angle (*i.e.*, between the two marks on two tires) can change over time along with the original fluorescent series x(t) and be computed for the fluorescent series on each cell by a Fast Fourier Transform [17]. The range of values for the Hilbert Phase by convention are usually taken to be from -π to π. If the process were $x(t) = A\cos(\omega t + \phi)$, then we could calculate the Hilbert Phase:

$$F^H(t) = tan^{-1}\frac{\tilde{x}(t)}{x(t)} = tan^{-1}\frac{A\sin(\omega t+\phi)}{A\cos(\omega t+\phi)} = tan^{-1}\tan(\omega t+\phi) = \omega t+\phi$$

In this case of a sinusoidal process the Hilbert Phase has a simple linear relation with time with the y-intercept being the phase shift ϕ and with the slope being the frequency ω.

To visualize the relation of the process x(t) and its replica $\tilde{x}(t)$ this pair of values is used to form a complex number of the form $(x(t), \tilde{x}(t))$, where x(t) is the real part and $\tilde{x}(t)$ is the imaginary part in the complex plane at time t (Figure 1C). This pair as a function of time is sometimes referred to as the analytic signal [16]. As time t advances, the curve $(x(t), \tilde{x}(t))$ traces out a cycle in the complex plane about its origin (Figure 1D). As the curve approaches -π or π, it tends to have discontinuities (Figure 2). To stitch together these discontinuities, the Hilbert

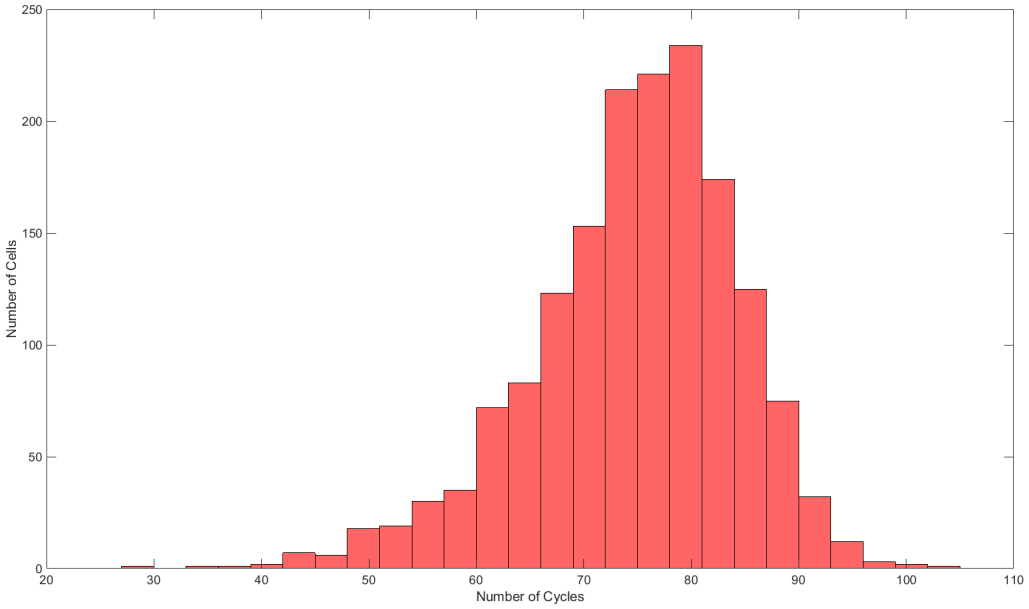

Figure 3. There was considerable variation in phase $M^C(t_1,t_0)$ over ~1,591 cells. The phase $M^C(t_1,t_0)$ was computed from t = 0 to t =281 h and is for ~1,591 cells that have no neighbors in a droplet. The mean and standard deviation of this histogram of phases for single cells were 74 cycles and 10 cycles, respectively.

Phase $F^H(t)$ is continuized as described in Materials and Methods and shown in Figure 2 (in red).

The continuous Hilbert Phase is denoted by $F^C(t)$ A plot of this average continuous Hilbert Phase with an average (over cells) of the original process is shown in the middle panel (Figure 2 in red). With time in the z-direction and the complex plane extending in the x- and y directions, the curve (x(t), $F^C(t)$) appears as a spiral (or tornado) in time (shown below) (see Figure 1D).

MATERIALS AND METHODS

Data. Fluorescent measurements were made on cells with a mCherry recorder gene attached to a *clock-controlled gene-2* (*ccg-2*) promoter in strain MFNC9 of *N. crassa* [18]. Much of the data were published in an excel spread sheet for ~1,591 isolated cells, each measured every half hour over ten days [7]. Cells are across columns; time is down rows. The fluorescent data were Rhodamine B normalized to control for uncontrolled periodic and aperiodic factors and detrended [1]. Subsequently, improved cell tracking slightly increased the data set to 1,644 cells. All data were loaded into a MATLAB (The MathWorks, Inc., Natick, Massachusetts, USA) workspace publicly available via GitHub at https://github.com/XiaoQiu2019/Matlab-code-for-phase-paper. Running input_data.mat and then plotcode.m will generate the figures with data in this manuscript.

Calculating Phase. The calculation of phase is presented [1]. To make this manuscript self-contained, the calculation of phase is detailed again. First a replica $\tilde{x}(t)$ (of the process x(t)) is created using the Hilbert Transform (available in MATLAB):

$\tilde{x}(t) = PV\frac{1}{2\pi}\int_{-\infty}^{\infty}\frac{x(t)}{t-\tau}d\tau$, where the integral is calculated in the principal value sense [19]. This replica $\tilde{x}(t)$ is 90 degrees out of phase with the original process; moreover, it is uniquely specified by the process when it exists. In that the replica $\tilde{x}(t)$ is purely imaginary in Figure 1C and completely out of phase with x(t), it can be derived from the original x(t) using the convolution theorem:

$\tilde{x}(t) = -iFT^{-1}(FT(x(t)))$,

where FT denotes the Fourier Transform, where FT^{-1} is the inverse Fourier Transform and where $i=\sqrt{-1}$. This relation is how the replica is computed with the commands fft and ifft in MATLAB [17].

The Hilbert Phase is the phase angle between the original process x(t) and the replica $\tilde{x}(t)$:

$$F^H(t) = tan^{-1}\frac{\tilde{x}(t)}{x(t)}$$

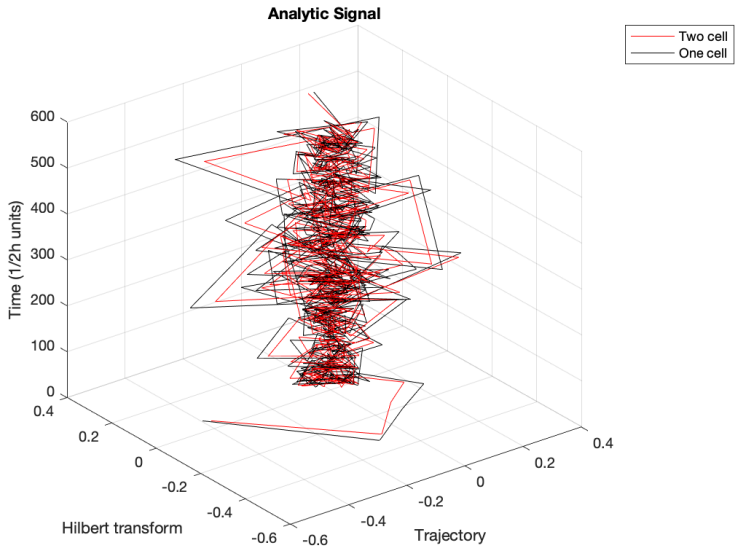

Figure 4. The curve (or analytic signal) (x(t), $\tilde{x}(t)$) spiraled over the complex plane for 2-cell (red) and 1-cell (black) trajectories over time with more phase variation in the 1-cell trajectories. The (x,y) plane is the complex plane. The vertical dimension is time. The trajectory of fluorescence x(t) was detrended after Rhodamine B normalization. The red tornado is an average over pairs of cells in the same droplet, and the black tornado is an average over single cells in a droplet [1].

As an example, if the process x(t) were $A\cos(\omega t + \phi)$, then the Hilbert transform would be $A\sin(\omega t + \phi)$. It follows that the Hilbert Phase reduces to $F^H(t) = \omega t + \phi$. As the Hilbert Phase passes near -π or π, there are usually discontinuities in the Hilbert Phase. To surmount this problem the Hilbert Phase was continuized. The continuous Hilbert Phase is defined recursively by:

$$F^C(t+1) = F^C(t) + m^C(t)2\pi \quad ,$$

where time t is an integer value indicating the number of elapsed half hours in each ~ten day experiment (containing ~480 half hour time points). The multiple $m^C(t)$ was chosen to minimize the following differences with respect to $m^C(t)$:

$$Df_m = \left| F^H(t+1) - F^H(t) + 2\pi m^C(t) \right|$$

This was done by the MATLAB code accessible in GitHub.

With the continuous Hilbert Phase $F^C(t)$ in hand, then the phase $M^C(t_1,t_0)$ in cycles can be calculated:

$$M^C(t_1,t_0) = \frac{\left[F^C(t_1) - F^C(t_0) \right]}{2\pi} \quad ,$$

where the divisor of 2π ensures that the phase $M^C(t_1,t_0)$ counts cycles completed by the continuous phase angle $F^C(t)$ and where the subtracted quantity acts like a generalized phase shift ϕ to remove the phase differences at the beginning of an experiment. The phase $M^C(t_1,t_0)$ in cycles varies with the time interval of the experiment. Allowing the phase to vary with time t_1 permits the examination of phase synchronization between cells as illustrated in the next section.

Analysis. All analyses were done by MATLAB or a Python script publicly available in GitHub at https://github.com/XiaoQiu2019/Matlab-code-for-phase-paper.

RESULTS

Phase Variation in single cells. It is useful to consider whether or not the continuation of the Hilbert Phase has any effect. The original Hilbert Phase $F^H(t)$ and continuous Hilbert Phase $F^C(t)$ were computed for a single cell and for each of the ~1,591 single cell trajectories and averaged (Figure 2). A Hilbert Phase $F^H(t)$ for a single randomly selected cell is shown on the top panel and is extremely ragged as it approaches -π or π, but the continuous curve $F^C(t)$ smooths over the discontinuities. The Hilbert Phase $F^H(t)$ is an angle and confined to the interval from -π to π (as shown in the lower panel of Figure 2). The average trajectory of the continuous Hilbert Phase $F^C(t)$ (middle panel) is smoother over time than the average of the original Hilbert Phase $F^H(t)$ too and is a measure of time for a periodic process [20]. The average Hilbert Phase $F^H(t)$ is also ragged due to the stochastic intracellular variation in each cell [9] as well as the

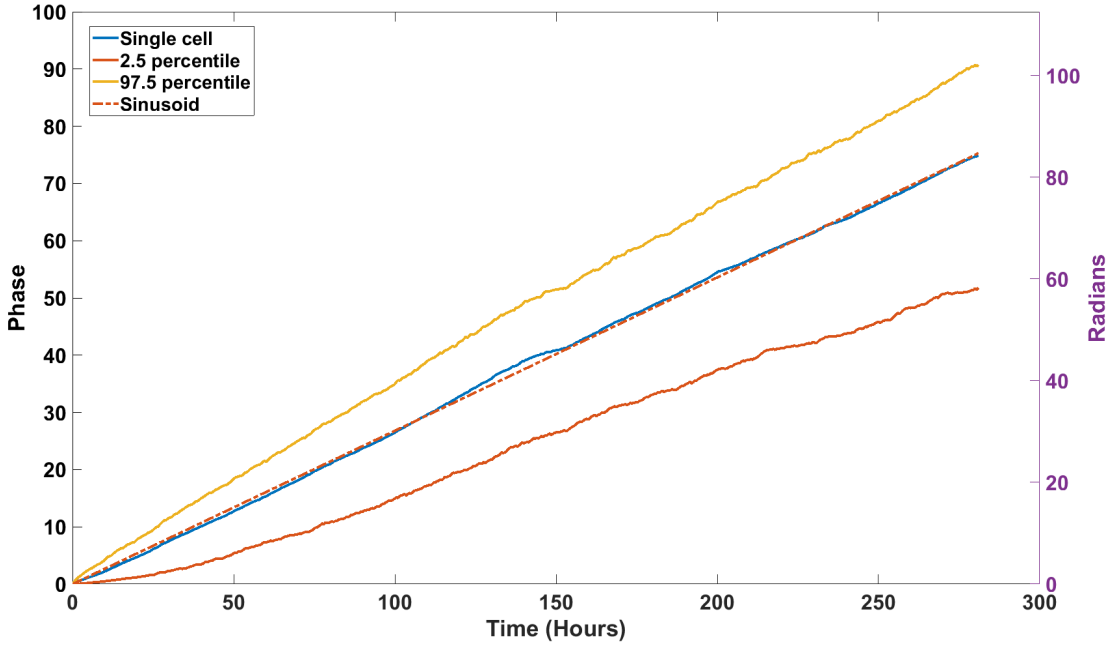

Figure 5. The phases of a sinusoid (in red) and that of the data (in blue) were similar, implying the oscillations are approximately sinusoidal. The average frequency ω = 0.3014 was used in x(t) = *A*cos($\omega t + \phi$) to compute the phase (in red). The average frequency ω was computed from fitting x(t) = *A*cos($\omega t + \phi$) individually to ~1,591 cell trajectories by Least Squares using a Python script. The percentiles in yellow and orange are shown about the mean phase.

discontinuity in $F^H(t) = tan^{-1}\frac{\tilde{x}(t)}{x(t)}$ at π and -π. The distribution of Hilbert Phase as a consequence appears non-uniform on the unit circle (Figure 2).

Each cell may have its own oscillator [1]. The phase $M^C(t_1,t_0)$ in cycles as defined above can then be calculated for each member of a population of ~1,591 isolated oscillators in *N. crassa* (Figure 3) [1]. The reported phases $M^C(t_1,t_0)$ are in cycles completed in the ten day interval of the experiment. The cycles completed on average are visible in Figure 2. As can be seen, there was substantial variation in the phase $M^C(t_1,t_0)$ of the oscillators as measured in cycles (Figure 3). At the single cell level each cell is marching to a different drummer. This raises the question of how cells synchronize to generate the coherent behavior at 10^7 cells.

Cellular clocks are fundamentally different from circadian clocks at the macroscopic level. Overlaid on the circadian rhythm is high frequency noise from the cell, much like an hour hand added on to the tolling of bells at the end of a day. The Hilbert Phase and Phase in cycles $M^C(t_1,t_0)$ count all cycles from all harmonics. As shown earlier an examination of the periods of cellular oscillators has demonstrated a major circadian harmonic [7] accounts for one cycle per day. The remaining cycles come from the high-frequency intracellular noise [1]. For example, the high frequency noise in a cellular clock allowed it to complete over seven cycles per 24 hour day (Figure 3).

Social environment of cells and its effect on phase. In the original experiments cells could be placed in the same droplet or in different droplets using a microfluidics device over ten days to observe circadian rhythms, thereby placing cells in different neighborhoods or "social environments" [1]. This feature of the experiments also allowed the examination of cellular communication between cells in droplets or neighborhoods. Those in the same droplet are within 74 microns [21] of each other and have the opportunity to communicate and hence perhaps synchronize. Those cells in different droplets have no opportunity to communicate and continue marching to their own drummer. It would be interesting to know whether or not 2-cell droplets have a different phase trajectory than 1-cell droplets. The tornado plots of the analytic signal (x(t), $\tilde{x}(t)$) are shown (Figure 4). The red tornado is an average over 568, 2-cell trajectories in the complex plane; the black tornado is an average over 1,644 single cell trajectories. While the 2-cell average trajectory very closely tracked the average 1-cell trajectory, the average 2-cell trajectory was consistently closer to the origin than the average 1-cell trajectory. This suggests that the 2-cell phase trajectories of the analytic signal behave differently than 1-cell phase trajectories, but this is analyzed in more detail with synchronization measures derived from the

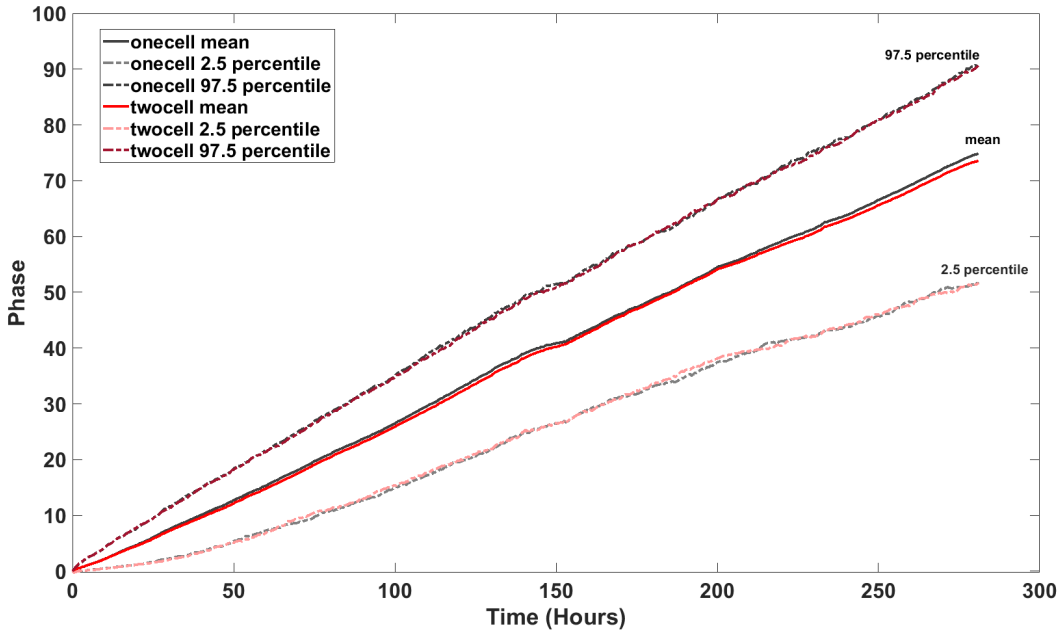

Figure 6. The average continuous Hilbert Phase $F^C(t)$ for 568 droplets each with 2 cells in one droplet (in red) is plotted against time differs from the average Hilbert Phase $F^C(t)$ for 1,644 cells each isolated in a single droplet and never having known neighbors. There were a total of 568 curves being averaged in the first case and 1,644 curves, in the second case. The 2-cell droplets are coded in red; the 1-cell droplets are coded in black. Percentiles are dotted lines with red-dotted lines belonging to the 2-cell droplets and with black-dotted lines belonging to the 1-cell droplets.

Hilbert Phase in the next section.

Phase as a function of time. Another feature of the phase plots is that they are a function of time. Both the continuous Hilbert Phase $F^C(t)$ and the phase $M^C(t_1,t_0)$ in cycles are plotted as a function of time t_1 with $t_0 = 0$ averaged over all ~1,591 cells (Figure 2 and Figure 5). The phase $M^C(t_1,t_0)$ in cycles resembles the Hilbert Phase, but passes through the origin in Figure 5. As a basis of comparison, the phase in cycles $M^C(t_1,t_0)$ for the data without a sinusoidal assumption and phase in cycles $M^C(t_1,t_0)$ for a sinusoid $x(t) = A\cos(\omega t + \phi)$ are plotted as a function of time in Figure 5. The parameters in the sinusoid were determined by least-squares for all 1,591 cells. As can be seen, the phase curves of the cells are approximately sinusoidal (Figure 5).

If the cells are in different droplets and unable to communicate, the expectation is that these cellular oscillators may drift out of phase with respect to each other. To test this hypothesis, the 2.5 percentile and 97.5 percentile curves about the mean phase of ~1,591 isolated cells (Figure 5) was computed over time. As expected, the percentiles drifted away from the mean over time. Incidentally the percentiles provide support for the phase curve (dotted) of the sinusoid not being statistically different from the average phase curve (in blue) of isolated cells. The phase curve for the sinusoid is found between the percentiles of the average phase curve for the cells.

The Hilbert Phase curves can also be used to examine the synchronization of cells, where phase may change with time. Consider 1,136 cells in droplets with 2-cells per droplet. How do these 568 (=1,136/2) pairs of droplets compare in phase with 1,644 cells that have never known neighbors? The average Hilbert Phase for 2-cell droplets steadily diverged from that of the 1-cell droplet in Figure 6 and was smaller than the average of the 1-cell droplets, as would be expected from Figure 4. Yet, the percentiles for both the 2-cell and 1-cell droplets still drifted apart with time, indicating an accumulation of phase variation with time even when cells have the opportunity to talk with each other.

Now consider 10 cells that have been placed in isolated droplets within the same data set [1] and never experienced neighbors. Also consider 10 droplets each with 10 cells that have lived together as roommates for 10 days within the same droplet. The average Hilbert Phase curves are shown for the isolated cells and 10 droplets each with 10 roommates (Figure 7). As can be seen, the average continuous Hilbert Phase for 10 cells in each of 10 droplets steadily diverged from the average of 10 singletons, who have never known neighbors. Their phases depend on the cell's social environment. Interestingly the 10-cell mean drifted outside of the 95 percent confidence

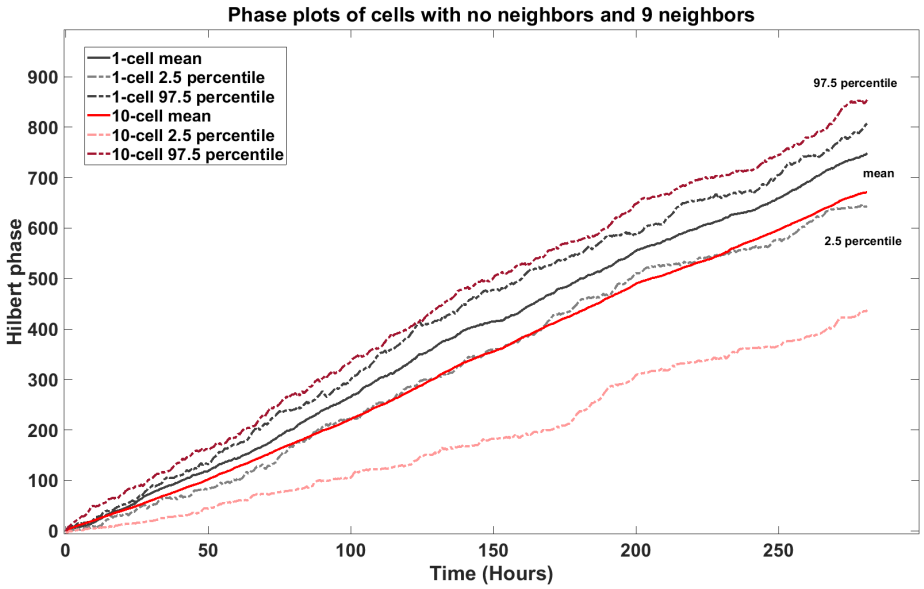

Figure 7. The average continuous Hilbert Phase $F^C(t)$ for 10 droplets each with ten cells in one droplet (in red) is plotted against time differs from the average Hilbert Phase $F^C(t)$ for ten cells each isolated in a single droplet and never having known neighbors. There were a total of ~100 curves being averaged in the first case and 10 curves, in the second case. The 10-cell droplets are coded in red; the 1-cell droplets are coded in black. Percentiles are dotted lines with red-dotted lines belonging to the 10-cell droplets and with black-dotted lines belonging to the 1-cell droplets.

band about the 1-cell droplet mean. Yet the phase variation also increases with time based on the percentiles of both 10 cell droplets and 10 singletons drifting away from the mean.

Phase also enters directly into the calculation of some synchronization measures [9]. One of the best studied synchronization measures is the Kuramoto order parameter(K) [22]:

$$K = \left\langle \left| n^{-1} \sum_{j=1}^{n} \exp\left(i\mathrm{F}_j\right) - \left\langle n^{-1} \sum_{j=1}^{n} \exp\left(i\mathrm{F}_j\right) \right\rangle \right| \right\rangle$$

The quantity $F_j = F_j^C(t_1)$ is the continuous Hilbert Phase for the jth oscillator with $t_1 = 480$ half hours. There are n oscillators in a particular social environment, and the brackets $\langle\rangle$ denote a time-average. As K approaches 1, the collection of n oscillators approaches being fully synchronized. As K approaches 0, the collection of n oscillators approaches no synchronization. A striking feature of biological oscillators is their social environment. Cellular oscillators may live together in a droplet or may be separated into separate droplets.

The Kuramoto K for the 1-cell and 2-cell droplets was computed as follows to examine the effect of sociality on oscillators. For the 1-cell droplets, the n in equation above was set to 1, and the K was computed for each isolated cell. From the resulting 1,644 K values the mean (0.0322) and standard error (0.0007) of K was computed. For the 2-cell droplets, the n in the equation above was set to 2, and the K was computed for each cell pair in a droplet. From the resulting 568 =1,136/2 K values the mean (0.7428) and standard error (0.0022) of K was computed.

It is useful to consider the effects of sociality on synchronization [9], and in particular to explain the difference in phase between 1-cell and 2-cell droplets in Figure 4 and Figure 5. As a control the Kuramoto K was calculated on all 1,644 1-cell droplets with the resulting K = 0.0322 +/- 0.0007, which is near zero as expected. Then the Kuramoto K was calculated on all 1,136 cells with n =2. The Kuramoto K for each pair of cells was then averaged over all droplets to yield K = 0.7428 +/- 0.0022. The conclusion is that for the 2-cell droplets cells within droplets are highly synchronized relative to the negative control provided by the 1-cell droplets, explaining the phase difference between 1- and 2-cell droplets in Figure 4. A z-test of the difference was highly significant

$$z \cong \left(.7428 - .0322\right) / \sqrt{\left(6.737X10^{-4}\right)^2 + \left(.0022\right)^2}$$

= 314.13, (P < .00001). A Wilcoxon Rank Sum test of the two unpaired samples of Kuramoto K's for 1-cell and 2-cell droplets was also highly significant (P < 0.00001).

Alternatively, a more conservative test is to create a sample of 568 pairs drawn randomly with replacement from the 1,644 cells in 1-cell droplets. With n=2 for the artificially generated pairs, in which strangers are neighbors, the average Kuramoto K for each pair of isolated

cells was K = 0.6853 +/- 0.0015. A z-test of the difference from cells that have truly experienced another cell was highly significant with z = 21.89 (P < .00001). The Wilcoxon Rank Sum test of the two unpaired samples of Kuramoto K's for 2-cell droplets vs artificially created 2-cell droplets was again highly significant (P <.00001). Other synchronization measures that reflect the sociality of the oscillators could be used to test for synchronization as well [9].

DISCUSSION

The notion of phase used here has been in use since the time of Huygens [23,24] in the description of coupled pendula right down to the present [16]. The notion of Hilbert Phase appeared early in the 20th Century [11]. All four phase measures (ϕ, $F^H(t)$, $F^C(t)$, and $M^C(t_1,t_0)$) are inter-related and graphically summarized in Figures 2 and 5. The first two measures (ϕ, $F^H(t)$) are defined on the circle, while the last two measures ($F^C(t)$, and $M^C(t_1,t_0)$) are defined on the nonnegative part of the real line.

There are several advantages to the notion of phase $M^C(t_1,t_0)$ in cycles described here. The phase $M^C(t_1,t_0)$ can be computed in an integrated way with the period and amplitude of a periodic process using the Fast Fourier Transform [17], in for example, MATLAB (see scripts on GitHub in Materials and Methods). The phase measure does not presume a linear relation with time as does the phase shift ϕ in a sinusoid. Using the phase shift as a measure of phase would be problematic in extrapolating to later times, if the phase were nonlinear in time, as it is likely to be when there is cellular synchronization. The phase measure $M^C(t_1,t_0)$ is functionally independent of the period and amplitude derived from the periodogram or "power spectrum" [1]. This phase measure represents new information about a periodic process not embedded in the amplitude and period [1].

The four phase measures are useful in combination. For example, the use of the phase shift in combination with the phase in cycles allows an assessment of whether or not a periodic process x(t) is sinusoidal (Figure 5). There is a generalization of the phase shift, namely the continuous Hilbert Phase $F^C(t)$ at time t = 0, which coincides with the phase shift, if the process x(t) were sinusoidal. This generalized phase shift is normally subtracted from the phase because often synchronization experiments are done to minimize this quantity – cells are started in a nearly synchronized state at the beginning of an experiment. Both the continuous phase $F^C(t)$ and phase $M^C(t_1,t_0)$ in cycles are smooth and provide information on when cellular oscillators synchronize (Figure 6). The original Hilbert Phase $F^H(t)$ is also in some sense closer to the data and can be plotted on a circle (Figure 2, lower panel). Some scientists also feel more comfortable with a notion of phase on the circle [20]. In this case the circular plot reveals a nonuniformity in the phase angle $F^H(t)$ over the circle. Finally, the phase can be used to evaluate various models for how cellular clocks synchronize [7]. When studying synchronization, the phase may be used to construct additional measures of synchronization [9] as illustrated here for the Kuramoto order parameter.

Cellular clocks are fundamentally different from circadian rhythms at the macroscopic scale – they have a high frequency hour hand in addition to the circadian cycle (Figure 3). Much of these high frequency cycles are generated by stochastic intracellular noise in reactions that go on within the cell [1]. There are multiple frequencies on which cellular clocks keep time, much like the two hands on a clock. This can be seen in the upper panel of Figure 2, in which cycles completed is greater than those generated by circadian oscillations present in single cell oscillators [7].

The Hilbert Phase has not only been used to study oscillators at the single cell level [1], but has been used to study other periodic biological processes, such as through monitoring the beating heart [25]. The Hilbert Phase and its derivative measure of phase here potentially provide useful new information about a variety of biological rhythms.

Acknowledgments: We acknowledge the support of the UGA Franklin/OVPR seed grant program, UGA College of Agricultural and Environmental Sciences, the ARCS foundation for support of E. Krach, NSF ECCS-1150042 from NSF Electrical, Communications and Cyber Systems, and NSF MCB-1713746 from NSF Systems and Synthetic Biology (SSB) and NSF Physics of Living Systems (PoLS).

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