PRC Bisection Tests

Daniel F. Kripke,* Paul Clopton,† Matthew R. Marler,*†
Shawn D. Youngstedt,* and Jeffrey A. Elliott*

*Department of Psychiatry
University of California, San Diego and
†San Diego Veterans Affairs Healthcare System

Correspondence:
Daniel F. Kripke, M.D.
University of California, San Diego 0667
9500 Gilman Drive
La Jolla, California 92093-0667, USA

telephone 858-534-7131
fax 858-534-7405,
email DKripke@UCSD.edu

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ABSTRACT

This manuscript presents a new method and computer program for evaluating phase response curves (PRCs). A phase response curve describes those phase shifts produced in an oscillator by stimuli applied at different initial phase-states of that oscillator. Analysis of variance (ANOVA) has often been used to evaluate the null hypothesis that resultant phase shifts are randomly related to the initial phase-state of the oscillator at which stimuli are given, but the PRC bisection tests presented here have several advantages. In the PRC bisection tests, we repeatedly cut in half the circular distribution of the initial phase-states of the oscillator when stimuli are given. We locate an optimal diameter which best bisects the circular distribution of phase responses into arcs of phase advance and phase delay. A **D** score reflecting the success of the best bisection is computed. The null hypothesis of a random distribution of phase responses by initial phase is tested with a Monte Carlo procedure, which bisects random combinations of phase shifts with initial phases, thus determining the probability of the null hypothesis that the observed **D** score was from a random distribution. The bisection procedure can also be used to examine whether stronger phase shifts are produced in one phase response curve than in a contrasting curve. Further, the bisection procedure yields an estimate of the inflection point of the phase response curve. Finally, a method is given to estimate the power of the PRC bisection procedure.

This note introduces a statistical program which implements PRC bisection tests for analyzing phase response curves (PRCs). Methods are given to determine if there is a PRC, to explore if one PRC is stronger than another, to estimate the PRC's angle of inflection with confidence limits, and to determine the method's statistical power for detecting PRCs.

What are phase response curves?

At any time, an oscillatory process has a phase-state (phi), which represents how far along its cycle the oscillator is at that particular time reference. When a stimulus is applied to a cyclic system such as a circadian oscillator, a shift in the phase state of that oscillator may result. The phase-shift (delta phi) is described as the difference between the initial phase-state (phi) of the oscillator minus its subsequent phase-state after the stimulus. When the phase-state (phi) of the oscillatory process regresses or moves backward after the stimulus, this produces a negative difference in the instantaneous phase called a delay, meaning that the stimulus has retarded the oscillator in its progress through its cycle. When the phase of the oscillator is sped up or shifted forward by the stimulus, this produces a positive difference called an advance, meaning that the stimulus has advanced the oscillator forward in its cycle. The change in phase commonly depends on the initial phase state of the oscillator at the time when the phase-shifting stimulus is applied. Thus, we can theoretically model and experimentally measure phase-response curves, which are the pattern of phase responses (i.e., phase shifts, delta phi) following stimuli, related to the initial phases in the oscillatory cycle (phi) at which the stimuli are applied.[1-4]

Phase-response curves (called PRCs) are commonly depicted with *XY* plots, where the ordinate is the phase response delta phi (delays negative and advances positive) and the abscissa is the range of initial phases (phi) from 0^o to 360^o. The abscissa of initial phases can also be plotted in "circadian time" or ct, as standardized in Johnson's atlas,[4] so that the intrinsic period (or tau) of the cycle is divided into 24 circadian "hours." In this usage, the initial phases on the abscissa then range from ct hour 0 to ct hour 24, beginning at subjective dawn (ct 0). Figure 1 presents a schematic example, using both degrees and circadian time as the abscissa.

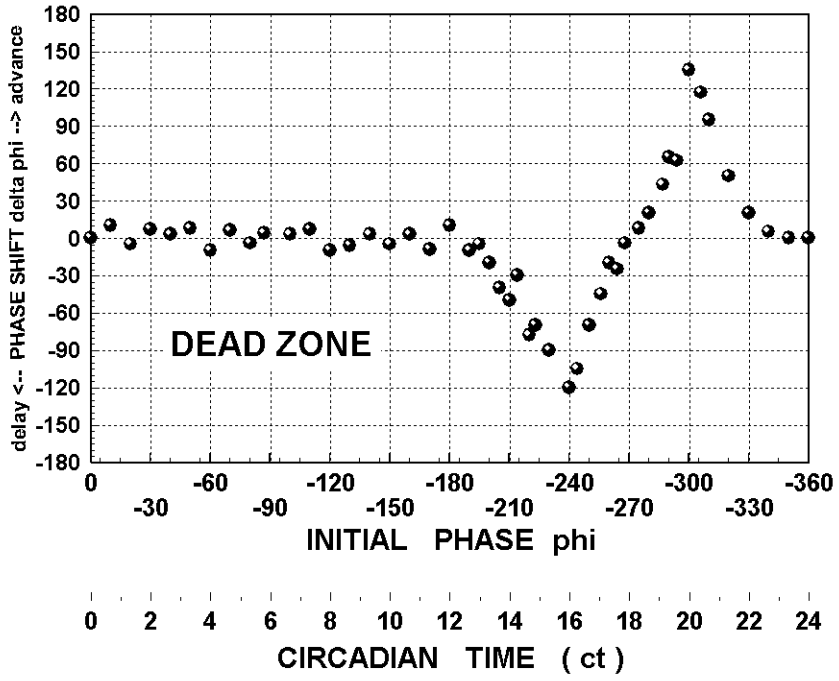

Fig 1: schematic diagram of a PRC, illustrating several points at which stimuli yield phase delays and several points which yield phase advances. The figure is intended to show an ideal PRC with little random error. The phase reference of the abscissa in angular degrees may be arbitrary, e.g., as in setting the subjective dawn equal to ct 0. Although we conceptualize circadian time as progressing in a clockwise direction, mathematically, positive angles are counter-clockwise. For this reason, clockwise progression may be labeled as negative degrees. An equivalent abscissa in circadian hours is also illustrated. The ordinate represents degrees of angular phase shift. The "dead zone" is an region where only negligible phase shifts occur.

Since the PRC depicts an intrinsically cyclic or circular process, the initial phase reference point may be arbitrary. PRCs can be plotted in many ways suggested by Winfree and others which reflect the circular distribution.[1,3,5,6] For example, since initial phases represent a circular distribution, they can be represented as points on a circle. The phase shifts may be represented by the vertical distance of each point above or below the initial-phase circle, as in Fig. 2.

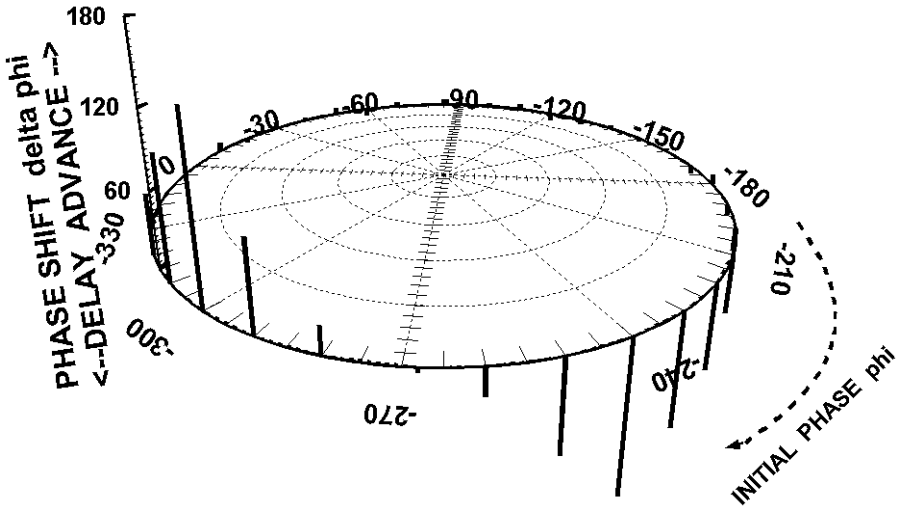

Fig. 2: A typical PRC is illustrated. Each vertical distance, illustrated by a line, represents the phase shift (delta phi) associated with a single stimulus at the initial phase (phi) represented by the angles around the circle. The vertical axis and scale represent the angular degrees of each phase shift. Points falling on the horizontal circle represent negligible phase shifts. The illustration shows an arc of consistent advance shifts (above) the circle of initial phases and an arc of consistent delays (below).

The phase-response curves which are found in circadian biology are extensively illustrated in Johnson's atlas.[4] In general, these curves have no more than one contiguous arc (range of initial phases) producing phase-advance shifts and no more than one contiguous arc producing phase-delay shifts. Moreover, most commonly, the arc of advances does not exceed 180^{o}, and the arc of delays does not exceed 180^{o}. There may be an arc where only negligible phase shifts occur (a "dead zone"), as well as a small area of undetectable phase shifts at the inflection from delays to advances (in Type 1 PRCs).[3,6]

Note that for several reasons, such as failure of the measurement method to completely account for a consistent decrease or increase in phase (which might result from free-running tau), there may be a trend towards measured delays or advances, resulting in an average shift which is greater or less than zero, even in the absence of stimuli. Regions of relative advance or delay must therefore be interpreted in comparison to the other portions of the phase response curve. For example, it is possible to imagine a phase response curve consisting entirely of delays, in which one portion of the curve demonstrates larger delays and another portion demonstrates lesser.

The statistical problem of recognizing phase response curves

In many cases, a descriptive plot of the PRC makes its amplitude and wave form obvious. It may be sufficient to indicate the confidence limits of the phase shifts produced at various initial phases. In other cases, particularly with stimuli producing low-amplitude Type 1 PRCs, it may seem uncertain if a given experimental stimulus produces phase shifts, that is, if the stimulus generates a PRC at all. In other words, when the experimental data consist of measured phase shifts following stimuli at various initial phases of the rhythm (e.g., a circadian rhythm), the experimenter may need to evaluate the null hypothesis that any phase shifts observed following the stimuli are randomly related to the initial phase at which the stimuli are applied. The alternative hypothesis is that there is some range of initial phases where resulting phase-shifts differ consistently from the mean shift. That is, there is some range biased predominantly towards advances or towards delays. It is assumed that each measured phase shift is independent of the other experimental data points, e.g., when each measurement is taken from a separate animal or plant.

Analysis of variance (ANOVA) has often been used to test for the presence of PRCs. Unfortunately, ANOVA may have unsatisfactory statistical power, because the true PRC effect is partitioned into several degrees of freedom. In addition, ANOVA may be difficult to apply rigorously if the experimenter cannot accurately predict the shape of the PRC, locating the arcs of phase advance and phase delay in advance. In particular, the point of inflection (where delays typically early in the subjective night yield to advances later toward morning) must be known in advance to use ANOVA efficiently, because otherwise, prospective choice of cell boundaries might place the largest advances and the largest delays within the same cell. However, it would be inappropriate to optimize the number of ANOVA cells or the boundaries for each ANOVA cell retrospectively. Finally, given the nonlinear nature of phase responses, the required assumptions for ANOVA including homogeneity of variance and Gaussian errors may often be worrisome.

Given these problems, it seems desirable to develop statistical tests which require less in terms of prospective prediction of the PRCs to be tested experimentally and less difficult assumptions.

The PRC bisection test for presence of a phase response curve

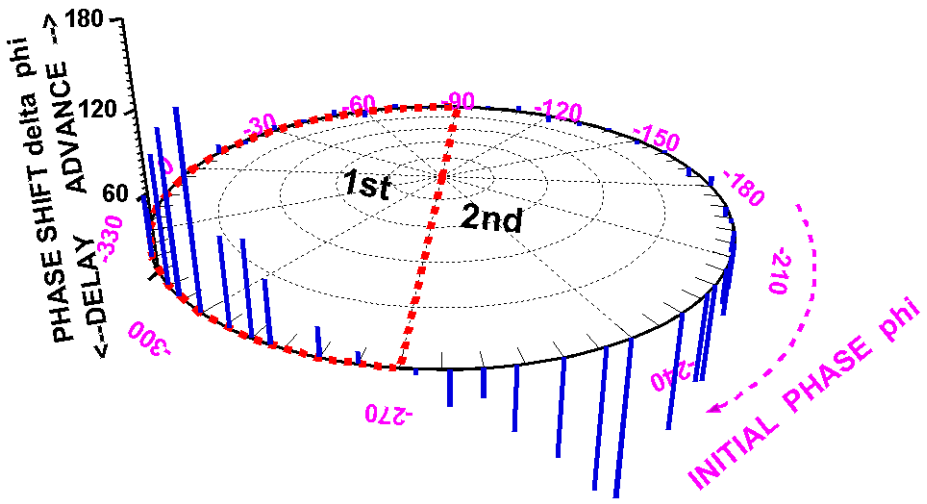

Fig. 3: The circular distribution of initial phases can be bisected into two hemicircles (180° arcs). The dividing diameter was selected so that in the 1st hemicircle (red dotted area), all shifts are positive (advances) or negligible. In the 2nd hemicircle, all shifts are negative (delays) or negligible.

To devise a new test of the null hypothesis that phase shifts are randomly related to initial phase, let us first consider some of the properties of PRCs. It is usually the case in PRCs (e.g., Fig. 1-2) that there is some phase-arc of initial phases spanning up to 180° in which the largest advancing phase shifts are observed, whereas in the other 180° of arc, the largest delaying phase shifts are observed. Fig. 3 shows how the 360° circular distribution of observed phase shifts can be divided (bisected) into two hemicircles or 180° phase-arcs which separate the advances and delays. Fig. 3 also illustrates that negligible (near zero) phase shifts will commonly occur within each hemicircle because of the dead zone. Of course, in real experimental data, there will be more experimental variation and measurement error, so that some mixture of advances and delays is likely to be observed in each hemicircle, without obscuring the overall trend.

To test the null hypothesis that the distribution of advance and delay phase shifts around the initial-phase-angle circle is random, we perform the following procedure on a set of experimental phase shifts. We test every possible bisection of the phase-angle circle, each bisection starting at an experimental initial phase and including all points clockwise within the next 179.99° degrees, thus dividing the experimental phase shifts into two 180° arcs of initial phases. When there are N data points, each representing a phase shift following a stimulus, there will be N possible bisections and sub-groupings of phase shifts, but some may have tied initial phases. Unless the initial phases represented by the data points are exactly equally spaced, there may be unequal numbers of points in the two bisected subgroupings. For each bisection and

subgrouping of initial phases, we compute the mean phase shift of each subgroup, and then we compute the difference between the mean shifts on each side of the bisection, so that

$$\mathbf{D} = \text{mean}[1^{st}\ \text{group}] - \text{mean}[2^{nd}\ \text{group}]$$

From the N possible bisections, we select that bisection producing the largest absolute value of **D**, which represents the most effective division of the phase shifts into advances and delays. Since the distribution can be bisected by a diameter starting from either side of the circle, if the absolute value of the most negative **D** is larger than the most positive **D**, we should select that most negative **D** and associate that |**D**| with the phase 180° opposite on the other side of the diameter. Now, we evaluate the significance of this optimal **D** score using a Monte Carlo method. We disassociate our experimental data into N initial phases and N phase shifts after stimuli. Then we produce 10,000 random combinations of the N initial phases with the N phase shifts after stimuli (using each value once). Each of these combinations has the same distributions of initial phases and of phase shifts, but the associations are randomized. For each of these random combinations, we repeat the bisection procedure, and we identify the largest difference for that random combination, $\mathbf{D_r}$. Thus, we obtain 10,000 values of $\mathbf{D_r}$ based on random combinations. The value of the p statistic, which is the probability of the null hypothesis that the observed distribution of advances and delays could have occurred randomly, is roughly the proportion of $\mathbf{D_r} > \mathbf{D}$. More precisely,[7] where N[$\mathbf{D_r}$] is the number of random combinations tested and N[$\mathbf{D_r}$>**D**] is the number in which $\mathbf{D_r}$>**D**,

$$p = (N[\mathbf{D_r}>\mathbf{D}] + 1) / (N[\mathbf{D_r}]+1)$$

To compute the p value of the null hypothesis and to determine the best bisection angle from a set of phase shifts, a Windows-compatible program is related to this document. First download the attached Zip file into a folder such as C:\Program Files\PRC\ and then unzip the download. Run the set-up program. Start the PRC program and click on the HELP button to learn the details of formatting input data and running the program. For simplicity, the initial phases may be entered as positive degrees rather than negative degrees to represent clockwise progression, and they do not need to be in order. When the program is run, a plot of the result is created and the p value of the null hypothesis appears in the program window, together with the angle of the best cut point. The HELP file also explains how the value of **D** can be read from the "first array."

Although this PRC bisection test may require millions of operations even for N of moderate size, it does not pose a difficult burden to contemporary computers. The PRC bisection test has several advantages:

- Since the PRC bisection test divides the data into only two parts (1 degree of freedom), it will generally be more powerful than ANOVA methods which divide the distribution of experimental phase-shifts into 4, 6, or 8 groups or more, with a larger number of degrees of freedom.

• The PRC bisection test does not require any prospective prediction of the shape of the PRC, i.e., what initial phases will result in advances or delays. Furthermore, the test should function adequately whether the PRC is Type 0 or Type 1.

• The PRC bisection test requires no assumptions regarding Gaussian distribution of the phase shifts or Gaussian distribution of errors.

• The PRC bisection test will function despite any uneven distribution of initial phases around the 360^{o}, even if not all initial phases (circadian times) are experimentally sampled, so long as a sufficient distribution of data points is collected from regions of the PRC producing contrasting phase shifts over at least 180^{o}. If the experimenter cannot be certain that the most contrasting regions of the PRC were adequately sampled, there is some potential for false negative results.

• The PRC bisection test will function if the range of initial phases which produces advances exceeds 180^{o} or if the range of initial phases which produces delays exceeds 180^{o} (though power may be reduced). The PRC bisection test will function even if all phase-shifts after stimuli are advances or if all phase shifts are delays, so long as one arc of initial phases produces more advance or delay than a contrasting arc.

As a general rule, the PRC bisection test can be applied in a satisfactory manner whenever the PRC consists of no more than one contiguous region (arc) of phase advances and no more than one contiguous region (arc) of phase delays. Exceptions to this model are rare in circadian biology, but they might occur in special situations such as instances where the circadian oscillator is split into two components. In situations where there is more than one region of advance or delay, the statistical power of the PRC bisection test may be impaired, and there will be increased risk of Type II errors.

Comparing the strength of PRCs

At times, an investigator may wish to contrast the strength of two PRCs resulting from different parts of an experiment. For example, the investigator may wish to examine if blue light or red light produces larger phase shifts in samples of animals. Similarly, an investigator may wish to test whether green light produces larger phase shifts in young than in old animals. Since the shape of the PRCs and the location of the initial phases producing maximal delay and advance will not necessarily be the same for the two experimental groups, contrasting two groups by the phase shifts produced at any single initial phase (phi) could be misleading. A test is needed comparing the overall phase responsivity of the groups, contrasting the overall magnitude of their PRCs.

The PRC bisection procedure offers an approach for comparing the overall strength of two or more PRCs. Consider again the optimal bisection of the phase circle illustrated in Fig. 3 and the derivation of the **D** score. The **D** score is an overall parameter reflecting the amplitude of the measured phase responses, summarizing the magnitude of both the advances and the delays. Note that subtracting the average negative delay shift obtained from the second

hemicircle increases **D**, since subtracting a negative quantity increases the difference. A bisection is optimal and the **D** score becomes maximal, not only when the average positive phase shift (advance) is large in the 1st hemicircle but likewise when the average negative phase shift (delay) is large in the 2nd hemicircle. We can extend this line of thinking to compare the distributions of phase responses between different experimental groups (See Fig. 4).

By computing the "Big Array," the PRC bisection program assembles the phase shifts from the first hemicircle (largely positive) and the phase shifts from the second hemicircle (largely delays) for each cut point, with the mean phase shift subtracted from each phase shift of the distribution (Fig. 4). Then the phase shifts from the second hemicircle are appropriately transformed by reversing the sign of that set of phase shifts. One can then select from the "Big Array" the distribution of N phase shifts appropriately transformed for the optimal cut point (the optimal initial phase of bisection). In the ideal situation where all phase shifts in the first 180^o are positive or negligible and where all phase shifts in the second 180^o are negative or negligible, all shifts in the bisection-transformed set will be positive or negligible. Analogous to the **D** score, the mean positive value of the bisection-transformed set of shifts will now represent the overall amplitude of the PRC. The usefulness of this bisection-transformed distribution is as follows. If we perform this procedure for two PRCs which we wish to compare, we can contrast the amplitudes of the two PRCs simply by examining whether the bisection-transformed distributions have different average amplitudes. The bisection-transformed distributions from the two PRCs can be simply contrasted with a nonparametric test such as the Mann-Whitney U, but in some cases the t test will be satisfactory. The question of whether paired tests (e.g., paired t tests) would be preferred needs further study. The bisection-transformed phase shifts can be pasted into any convenient statistical program for this purpose. Similarly, three PRCs or more can be contrasted using a test such as the Kruskal-Wallis nonparametric ANOVA.

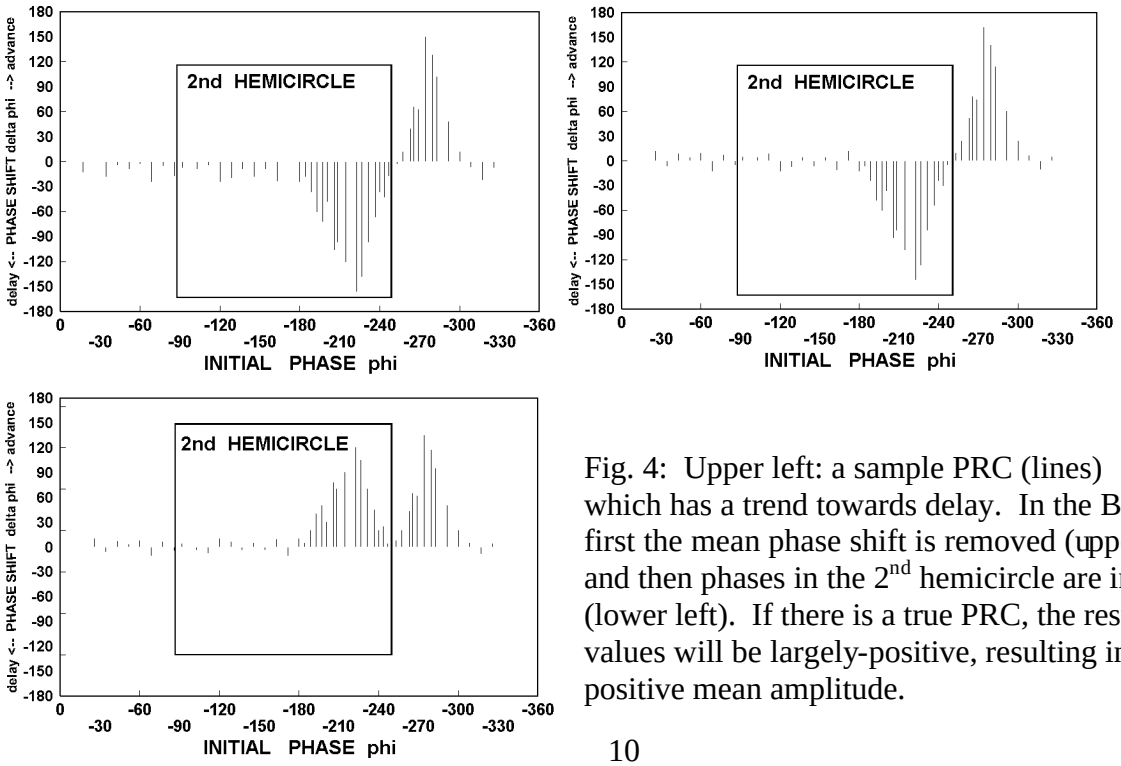

Fig. 4: Upper left: a sample PRC (lines) which has a trend towards delay. In the Big Array, first the mean phase shift is removed (upper right), and then phases in the 2nd hemicircle are inverted (lower left). If there is a true PRC, the resulting values will be largely-positive, resulting in a positive mean amplitude.

An important caveat must be explained in using these methods to examine the amplitudes of PRCs. Obviously, the magnitude of the **D** score and the average amplitude of a transformed distribution of phase shifts (Fig. 4, lower left) will vary if the initial phases of the data points are not evenly distributed around the phase circle. If a disproportionate number of stimuli were given in the subjective night during the interval of large delays or large advances, the mean amplitude will be increased. If a disproportionate number of stimuli were given during the subjective day or in the dead zone, a decreased mean amplitude will result. The mean amplitude is only an unbiased parameter when the distribution of initial phases is unbiased, a question easily examined with the Rayleigh Test.[8] Likewise, using the PRC bisection method to contrast the amplitude of two or more PRCs may be biased unless the stimuli were evenly (or randomly) distributed around the initial-phase circle.

If the investigator is willing to assume that the shapes of the PRCs from the experiments to be compared are identical and that the stimuli were given at analogous phases, the PRC bisection method might provide an unbiased comparison between groups even when the initial phases are not evenly distributed around the phase circle. Assuming identical shapes (but differing amplitudes) of PRCs would always be a bit daring. Kuiper's circular adaptation of the Kolmogorov Smirnov test, as described by Batschelet, is suitable for determining if the distributions of initial phases are equivalent.[8]

Identifying the point of inflection

In the typical Type 1 PRC, an arc of phase delays beginning in the late subjective day or early subjective night is followed by an arc of phase advance shifts spanning the late subjective night and early subjective day. We refer to the phase of this transition from delays to advances as the point of inflection. The transition from delays to advances may be gradual as in many low-amplitude (Type 1) PRCs, or it may be extremely abrupt (as in Type 0 PRCs, where it has been called the breakpoint). By finding the phase angle for bisection of the phase circle which produces the highest **D** score, the PRC bisection program will usually be making a nearly-optimal estimate of the phase of the point of inflection between delays and advances within the subjective night. It is true that the bisection procedure weighs the data from the entire phase circle, parts of which are remote from the point of inflection, but in a typical Type 1 PRC, the point of inflection and optimal phase for bisection will closely coincide because the opposite subjective day is a "dead zone" in which phase shifts are almost negligible. Therefore, the dead zone will contribute little to the location of the best cut point (phase angle) for bisection. The best angle of bisection may represent the best phase reference point or parameter for the overall timing of the PRC. The situation may be different for some Type 0 PRCs, particularly those in which there is little or no dead zone, that is, when the crossover from decreasing advances to increasing delays has appreciable slope and no intervening dead zone. In such cases, this mid-day crossover will also influence the computed best angle of bisection, whereas determination of the point of inflection (breakpoint) in the subjective night may depend on arbitrary switching of the sign of the phase shift to keep phase-shift magnitudes less than 180^{o}. For such Type 0 PRCs, there is little risk that the PRC bisection method would fail to detect a significant PRC, but the

best bisection angle will be untrustworthy as a timing marker, because it might be largely determined by the arbitrary assignment of sign to phase shifts of approximately 180°.

The best angle of bisection, called the "cut point" in the PRC program, may be read from the program window or from "array 1", as described in the HELP file. This is the best estimate of the point of inflection. The same data may be viewed on the graph provided.

To determine the confidence limits of the point of inflection, obtain the "Big Array" as described in the program HELP. As mentioned, the "Big Array" gives for each possible cut point, the distribution of the N phase shifts corresponding to the **D** score obtained with that cut point. By comparing the distribution of phase shifts at the optimal cutpoint with the distribution for each other cut point (with a t test or non-parametric test, for example), which cut points give a significantly different **D** score can be ascertained at a given alpha (level of significance). To allow for the fact that the phase circle can be bisected from either side, in making these computations, it is appropriate to reverse the sign of all "big array" phases for cutpoints which produce negative **D** scores, associating the resultant distribution with the cut point 180° across the phase circle. Cut points yielding significantly different **D** scores from the optimal cut point are outside the phase confidence interval for the best cut point.

Estimating the power of the PRC bisection test

Frequently, an investigator may wish to estimate the power of the PRC bisection test for detecting a hypothetical result, perhaps to plan an experiment or perhaps to evaluate the confidence of an outcome indicating the absence of a significant PRC. Power testing can also be performed with a Monte Carlo method, providing that the investigator is willing to prospectively specify a general description of the PRC which the test will attempt to detect. To perform the power test, the investigator must predict the magnitude of the advances and delays in the PRC which the procedure will aim to detect, the general shape of the PRC curve, the number of points to be tested, and the likely distribution of the initial phases of the points tested. In addition, the error variability (standard deviation) of the experimental measurements of phase shift (delta phi) for a given initial phase (phi) must be predicted. Then, the power test simply synthesizes 100 model PRCs, adding error randomly using the specified magnitude of random error, and computes the PRC bisection test for each synthetic model. The percentage of model tests with significant p is the power of the test to detect the hypothesized curve.

The power test will be most sensitive to the number of points measured, the predicted amplitude of the PRC, the reliability of measurement (standard deviations), and the percentage of the 360^{o} predicted to contain dead zone. The power test will not be sensitive to uncertainty in predicting the location of the inflection point. Moreover, the test will not be very sensitive to the predicted shape of the PRC, since within the hemicircles defined by the optimal bisection, where the largest phase shifts appear does not affect **D**.

Conclusions

This note explains a new statistical approach to phase response curves. The related Windows computer program implements these tests in a usable laboratory version, not so polished as a commercial program might be.

As yet, we have only limited experience with the PRC bisection tests and the computer program. We will be grateful for the critique and advice of colleagues in trying to develop this approach.

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