Synchronization between beating cilia

Larisa Gheber and Zvi Priel
Chemistry Department, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

ABSTRACT A novel quantitative parameter is proposed to define and measure the degree of synchronization between two small ciliary areas. These areas can be close to or far from one another. The Pearson correlation factor is used to define the degree of synchronization by a single number. This approach is based on a computerized, dual photoelectric method which simultaneously measures the scattered light from two small areas on the ciliary epithelium or its tissue culture. The measurements were performed on tissue culture from frog's palate epithelium. It was found that: (*a*) the degree of synchronization decreases as a function of distance; (*b*) the correlation is fairly high even at relatively large separations, when measured on the same patch; (*c*) on a given patch the synchronization factor is independent of the direction of the metachronal wave; (*d*) close disconnected ciliary cells exhibit fairly high correlation; (*e*) disconnected randomly choosen ciliary cells at relatively large separation distances exhibit relatively low correlation, smaller by a factor of 2 than the correlation factor at the same distances when measured along the metachronal wave; (*f*) the average frequencies' ratio and the metachronal wavelength can be used as first-order indicators of ciliary synchronization; (*g*) there is a spread of metachronal wavelengths even over a single well-organized patch.

INTRODUCTION

The main function of cilia is to make biological transport possible. For this task to be efficiently performed synchronization between beating cilia is essential. This is particularly obvious when the close spacing of cilia relative to their length on most ciliated cells is considered. As far as we know, a method that defines the synchronization between beating cilia has not yet been introduced and consequently this important property has not been measured. The existence of synchronization is manifested by the metachronal wave. Clearly, synchronization is a necessary condition for the appearance of wave motion in space and time. However, the metachronal wave may come about with varying degrees of synchronization. Terms such as weak or strong metachronism are frequently used in ciliary studies to describe qualitatively the observed degree of ciliary synchronization. The aim of this study is to define a quantitative measurable parameter that will describe the degree of synchronization between beating cilia.

The biological or physiological importance of this parameter is obvious when the transport capability of ciliary system is analyzed or an attempt to measure communication between cilia is made. Changes in the degree of synchronization between beating cilia will strongly influence their ability to transport material even when all other parameters (such as frequency, amplitude, etc.) will remain constant. A total dissynchronization of cilia on the epithelium will result in materials not being transported at all regardless of other ciliary activity. It was found (Priel, 1987) that on the frog's palate the metachronal wave velocity is larger by a factor of 2 than the particle velocity. One of the reasons for the reduction in particle rate velocity may be due to the synchronization's being only partial. The mechanism of ciliary synchronization is still an interesting and open problem in ciliary study. Several mechanisms have been suggested for different species and organs ranging from nervous control for the frog's oropharyngeal cavity (Seo, 1931), membrane activity in trachea (Gosselin, 1966), neurotransmitters or local mediators which diffuse below the basement membrane to the ciliated cell, and membrane potential in the oviduct (Brenner, 1969). As can be seen, a general and common control mechanism for ciliary activity was not found and most of the mechanisms suggested above were not proven. Moreover, it seems that there is a general tendency to believe that control decreases in the higher forms of life, and that ciliary beating is autonomous in mammals (Gray, 1928; Kinosita and Murakami, 1967; Sleigh, 1962; Aiello, 1974). Therefore, the problem of how a synchronized metachronal wave forms from autonomous beating cilia is still a puzzle. Several mechanical and hydrodynamic mechanisms have been suggests (Gray, 1930; Aiello and Sleigh, 1972) but to the best of our knowledge they too were not proven. It is our belief that it was the absence of a quantitative method of

0006-3495/89/01/183/09 $2.00

measuring the degree of synchronization between beating cilia that hindered the systematic study of the mechanism of ciliary synchronization.

Recently, a method was developed (Eshel and Priel, 1987) that makes it possible to measure (among other things) the degree of synchronization between beating cilia. It is based on simultaneous measurement of scattered light coming from two points on the ciliary epithelium. The distance between the two points can be varied from zero to hundreds of micrometers by steps of 0.5 μm.

We define the synchronization factor between two relatively small ciliary areas at a given distance using bivariate correlation analysis (Blalock, 1972). This correlation provides a single number which summarizes the relationship between the two signals. This correlation coefficient indicates the degree to which variation in one variable is related to variation in another. In other words this correlation coefficient will describe quantitatively the degree of synchronization between two small ciliary areas a given distance apart.

It was found that the correlation factor is a sensitive and reproducible parameter. The synchronization is found to gradually decrease as a function of distance between the two areas. On a single ciliary cell a high degree of synchronization ($\bar{P} \geq 0.9$) was observed. Even at distances as large as 100 μm, when measured on a given patch, there exist a relatively high correlation ($\bar{P} \geq 0.6$). The synchronization is independent of the direction of the metachronal wave in the vicinity of the metachronal wave.

MATERIAL AND METHODS

The experiments were carried out on locally supplied frogs (*Rana ridibunda*). Ciliary tissue cultures were prepared from the palate in a manner described previously (Eshel et al., 1985). Before the measurement the medium over the tissue culture was changed several times, until the concentration of mucus was reduced to below several parts per million (ppm), which is the limit of sensitivity of our dynamic light scattering spectrometer (model 4700, Malvern, Malvern, UK). It is important to note that the dynamic light scattering method is extremely sensitive to high molecular weight substances of which the mucus is composed. After this treatment, usually no detectable increase in mucus concentration was observed over several hours, which was enough to complete one set of experiments.

The preparation was placed flat on the stage, cilia uppermost, with the light coming from above (100 Tungsten-Halogen lamp fed from a stabilized homemade DC power supply) and passing through the epithelium. An inverted microscope (model IMT, Olympus Optical Co., Tokyo, Japan) was used, so that the objective beneath the specimen would be focused on the ciliated surface. Optical fibers (50-μm cross section diameter) were placed in the focal plane of the oculars. One fiber was fixed exactly in the center of the field (model 700-10-36a, EGGG Gamma Scientific, San Diego, CA) and the other (700-10-62, Gamma Scientific) could slide along an axis lying in the focal plane, by means of a micrometer screw. The orientation of this axis could be changed simply by rotating the eyepiece. An objective of $\times 20$ corresponding to a measured area of 4.9 μm^2 was used. Each fiber was connected to a separate photomultiplier (model 96358, EMI Electronics, Hayes, Middlesex, England). The photomultiplier outputs were further amplified (low-noise preamplifier model 113, Princeton Applied Research Co., Princeton, NJ) and also digitized into the memory of a microcomputer (IBM-XT) with a sampling rate of 360 Hz.

The two fibers were brought above the same location on the preparation in the following manner: first, some marking point was brought exactly below the tip of the fixed fiber, and next, the movable fiber was also adjusted above the same point by means of the micrometric screw. An area of beating cilia was then brought below the fibers and final corrections made for producing on the oscilloscope screen identical time-dependent-signals. On comparing visually the identity and the "0" phase between the two signals, an estimated accuracy of ± 1 μm is achieved in the determining the 0.

The signals with 0 distance between the two fibers were sampled and analyzed. The movable fiber was then moved along its axis (which was chosen as the one with the greatest phase gradient), in steps of 1 or 1.5 μm (± 0.2 μm), and the signals again sampled simultaneously.

The calibrations of the data acquisition system and the computer analysis were performed as described previously (Eshel et al., 1985). We adapted to the microscope a rotating stage with an accurate scale. The main feature of the stage was its rotation around the optical axis of the microscope. Calibration of the stage revealed that during a rotation of 180° it keeps its center better than 0.4 μm. Deviations of this order of magnitude are negligible for our purposes and, therefore, are ignored.

In order to establish the average phase difference versus distance, we use the Gorelik (1959) approach, which gives:

$$\cos(\phi_2 - \phi_1) = \frac{a^2 - (a_1^2 + a_2^2)}{2a_1a_2}, \qquad (1)$$

where a_1 and a_2 are the average amplitudes of the two signals respectively, a is the average amplitude of the arithmetic sum of the two signals and $\phi_2 - \phi_1$ is the average phase difference between the two signals. In order to calculate the phase difference between the two signals the following procedure was adopted: (1) Two signals each 40 s long are recorded simultaneously with a sampling rate of 360 Hz. (2) Each signal is divided into consecutive 1-s segments, creating 40 such pairs. (3) The phase difference for each 1-s pair is calculated according to Eq. 1, and averaged over the 40 segments. The analysis of Gorelik is statistical; a pair of 1-s segments which are composed of $2 \times 360 = 720$ experimental points constitutes an adequate sample for such an approach.

The optical method described above enables us to measure the wavelength and frequency of the metachronal wave (Eshel and Priel, 1987), and its wave velocity (Priel, 1987). Because of the relative easiness of the measurement, fairly large statistical ensembles can be formed, increasing considerably the accuracy of the measurements so that quite an accurate description of the metachronism in ciliary systems is obtained. The method, however, can not differentiate between

TABLE 1 Comparison between phase calculated by Gorelik's method ($\bar{\Delta}\varphi_G$) and the Pearson method ($\bar{\delta}\varphi_P$)

Δx	$\bar{\Delta}\varphi_G$	$\bar{\Delta}\varphi_P$
	degrees	
0	2.50	2.37
1.5	37.1	37.4
3	77.2	75.8
4.5	109.8	110.3
6	148.4	146.9
7.5	178.6	179.7

different types of metachronism (i.e., symplectic, antiplectic, etc.), which is one of the main drawbacks of this technique.

Formulation of the synchronization factor

In order to measure the degree of synchronization between two relatively small ciliary areas a given distance apart, we use the Pearson product-moment correlation coefficient, which is defined mathematically by:

$$P = \left(\sum_{i=1}^{N} (x_i - \bar{x})(y_i - \bar{y})\right) \cdot \left\{\left[\sum_{i=1}^{N} (x_i - \bar{x})^2\right]\left[\sum_{i=1}^{N} (y_i - \bar{y})^2\right]\right\}^{-1/2}, \quad (2)$$

where x_i = ith observation of variable x, y_i = ith observation of variable y, N = number of observations,

$$\bar{x} = \sum_{i=1}^{N} \frac{x_i}{N} = \text{mean of variable } x,$$

and

$$\bar{y} = \sum_{i=1}^{N} \frac{y_i}{N} = \text{mean of variable } y.$$

When there is perfect coupling P will be $+1.0$ or -1.0. A negative P does not mean poor coupling, rather, it describes an inverse relationship: as x increases, y decreases. A positive correlation means that x and y increase (or decrease) simultaneously. When the linear regression line is a poor fit to the data, P will be close to zero. Indeed, the value of zero denotes the absence of correlation.

In our case, these two signals stem from different location along the metachronal wave they are in general out of phase. The phase difference between the two signals will reduce the Pearson correlation factor. The aim of this work is to introduce a parameter that defines the degree of correspondence between patterns of beating cilia regardless of the phase differences between them. Moreover, the phase difference can be measured independently as was demonstrated by us (Eshel and Priel, 1987). In order to achieve this goal we have to introduce a correction into Eq. 2. The simplest method of correction, is to mathematically change the phase of one of the signals until P is maximized. By introducing this correction negative P values are eliminated and P now changes between 0 and 1 (see Eq. 2). We have calibrated this method using two synthesized signals with a given phase difference between them. It was found that a maximum correlation factor (P = 1) corresponds to zero phase difference between the two signals. This also turned out to be the best procedure to evaluate the phase difference between two locations. The comparison between the phase differences estimated by this procedure and by the Gorelik method used by us previously (Eshel and Priel, 1987) shows good agreement (Table I). The relatively high correspondence between such two entirely different methods indicates that all the requirements of the bivariate correlation analysis are fulfilled, and that, our phase correction is valid.

In order to calculate the Pearson correlation factor, the following procedure was adopted. Two signals were measured simultaneously for 40 s and sampled at time intervals of 1/360 s (creating an esemble of 2 × 14,400 points). Then 40 Pearson correlation coefficients, one for each second, were calculated for the pair of signals. The 40 values obtained were then averaged to obtain a single number. When the two signals were taken from the same location on the ciliary tissue the phase difference between them is zero and Eq. 2 can be applied to calculate the correlation coefficient without need for a phase correction. The statistical sample is fairly large and a reliable correlation factor may be expected. The measurement is then repeated using two relatively small ciliary areas at a well-defined separation. Such a measurement will yield two semiperiodic signals which may have a phase difference between them if taken along the metachronal wave. The phase difference will be zero if taken parallel to the metachronal wave. This was shown by measuring the phase differences between beating cilia as a function of declination angle from the metachronal wave direction (Ovadyahu and Priel, 1989). As shown in Fig. 1 *a* the two signals may be shifted by a constant phase relative to one another. The Pearson correlation factor is, as explained above, affected by this phase difference and is maximum only when the phase difference is zero. To obtain this value we therefore, shift t, for one signal (Fig. 1, *b* and *c*) until a maximum value for P is obtained. The time shift which yields this maximum value is, of course, the phase difference between the two ciliary areas. As is well known both the frequency and the phase of ciliary motion are not entirely constant but change somewhat with time. For this reason the degree of synchronization and the factor P, between two ciliary areas, are expected to show some dependence on observation time. When shifting the time coordinate for one signal relative to the other it will, on the average, be easier to bring signal measured over short time to closely overlap one another than signals measured over long intervals. In other words, P is expected to decrease somewhat with interval length. Experimentally, we find P to remain constant for intervals between 0.3- and 2-s length and then to decrease slowly until, for a length of 40 s a 10% decrease is obtained. In view of these results we have adopted a 1-s interval as our standard one. Fast Fourier transform (FFT) analysis of ciliary beating yields similar results and the use of a 1-s window has become standard for such studies.

In principle, when the two signals are sampled from the same location (Δx = 0), the Pearson correlation factor should be 1 by definition. However, in our measurements P is always less than 1 ranging from 0.88

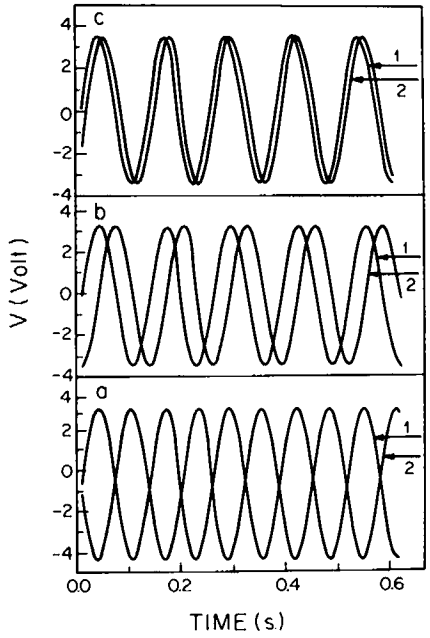

FIGURE 1 Schematic representation of phase correction accomplished by shifting one signal relative to the other in time. The Pearson correlation factor P increases from *a* to *c*. As the phase shift is further reduced P is maximized when the shift reaches zero.

to 0.99. This can be understood by remembering our inability to focus on exactly the same area with our two separate measuring systems. Although the overlap is fairly high, it is definitely not perfect. In addition, we are measuring these two signals by two completely different electronic systems (see Materials and Methods) which introduce uncorrelated noises and therefore reduce measured P value. The observed relatively high P values obtained for these conditions ($\Delta x \sim 0$, $0.88 \leq \bar{P} \leq 0.99$) provides evidence for the high overlap of focusing and high signal-to-noise ratio of the measuring systems. For a given experimental set (P vs. Δx) the noise and initial error in Δx are a constant factor which is not physically meaningful but may be different from one set of experiments to another. Therefore, in order to compare between different sets of experiments we normalized the results to give $P = 1$ for $\Delta x = 0$.

It is obvious that the Pearson correlation factor is dependent on the frequency difference if any between the two examined signals. The exact relation between them can be calculated analytically by assuming that the two signals are sinusoidal with frequencies f_1 and f_2. It is easy to show that for this case the correlation factor is given by

$$P = \frac{K}{2\Delta f} \sin 2\pi \Delta f, \tag{3}$$

where $\Delta f = f_1 - f_2$ and K – is a normalization factor. This analytical solution, however, does not include the correction introduced by us for phase difference. Simulation studies show that introduction of the phase correction causes P to depend less strongly on Δf than is predicted by Eq. 3. Therefore, in order to find the P dependence on Δf in our case, we have calibrated the system using a simulation in which two sinusoidal signals with different frequencies are substituted for the signals derived from the two ciliary areas. Such a simulation is valid when the time dependence of ciliary frequencies and phases are ignored but allows varying the frequency difference at will. The result of this calibration is given in Fig. 2. As can be seen at $\Delta f = 0.65$ Hz the Pearson correlation factor reaches $P = 0.5$. At the range of frequency differences from 0 to 1 Hz the relation between the Pearson correlation factor and Δf can be written as

$$\Delta f = \frac{1}{87} \arccos P \quad \text{(see } \textit{inset} \text{ in Fig. 2).} \tag{4}$$

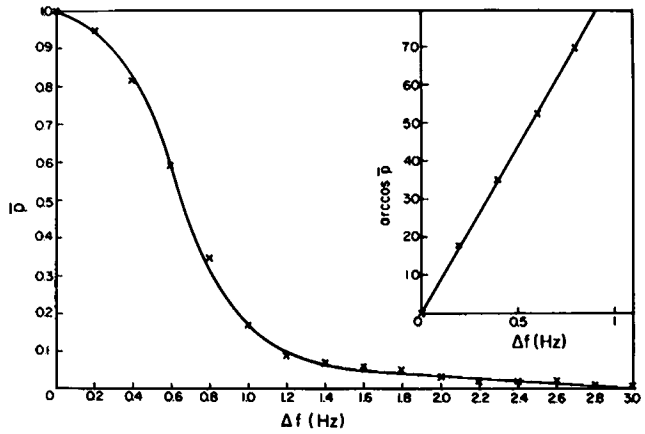

FIGURE 2 Pearson correlation factor as a function of frequency differences between two signals. The signals are derived from two separate electronic oscillators and are sampled and treated exactly as would signals obtained from cilia. (*Inset*) The linear relationship between arccos P as a function of Δf, Δf being limited to 1 Hz.

Using Eq. 4, it is possible to estimate the average frequency difference between the two signals from the measured Pearson correlation factor.

The suggested synchronization factor (P) is completely different from the term "line synchronization" which was introduced by Machemer (1974) and frequently used in the literature. Whereas the term line of synchronization defines a line of cilia beating with the same phase (uniphase line), we introduce a quantitative parameter that defines the degree of synchronization regardless of the phase difference between two ciliary areas.

To conclude, we have defined and discussed the Pearson correlation factor (Eq. 2) and introduced two corrections to the basic definition: (*a*) removal of the dependence of P on phase difference between the two signals and (*b*) normalization of the correlation to give $\bar{P} = 1$ at $\Delta x = 0$. We have outlined the procedure of using P in our study and described the dependence of the corrected P on Δf (Eq. 4).

RESULTS

Short-range synchronization

Typical behavior of the average Pearson correlation vs. distance is shown in Fig. 3 *a*. This figure represents a set of experiments for which the averaging has been performed, for each distance, on signals measured during 40 s. As can be seen the synchronization between beating cilia decreases as the separation distance between beating cilia is increased. Over the dimension of one ciliary cell the synchronization between cilia is fairly high ($\Delta x = 6$ μm, $\bar{P} = 0.89$). The average frequency as a function of location as measured by the moving optical fiber is given in Fig. 3 *b*. This representation, however, may be misleading since measurements at different Δx are taken at different times. Therefore, Fig. 3 *b* may also represent the frequency behavior as a function of time. Time dependence of ciliary frequency has indeed been observed (Kennedy and Ducket, 1981; Eshel et al., 1985; Zahm et al., 1986). In order to separate the time and distance dependence we divided the frequency observed at one area (f_1) by the frequency measured at the same time at

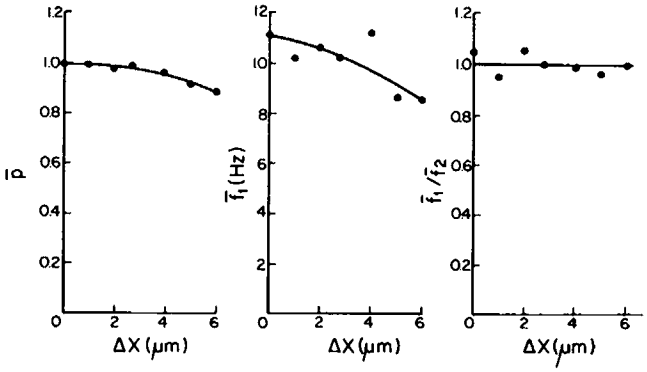

FIGURE 3 A typical short-range measurement of the Pearson correlation factor, frequency of one of the measured areas and ($\bar{f}_1/\bar{f}_2$) as a function of Δx.

the other area (f_2). If the changes in frequency are correlated over the measured distances, then the same variation in frequency has to occur in both areas during the same time. Therefore, dividing the one frequency by the other, sampled at the same time should yield, regardless of the distance between them, $f_1/f_2 = 1$. In contrast, if the frequencies sampled from different places are different and/or if they are uncorrelated, then $f_1/f_2 \neq 1$. It was observed that within the dimension of one ciliary cell the average frequencies are the same, to within experimental error ($\pm 5\%$) (see Fig. 3 *c*). The parameter f_1/f_2 may also be used as an indication of the correlation between ciliary motion at different areas. This parameter, however, is a relatively crude one as compared to the Pearson parameter, since f_1/f_2 is based on the dominant frequencies only whereas P is affected by the entire spectra of both signals. Therefore, it is not surprising that while $\bar{P}$ vs. Δx is a decreasing function, $\bar{f}_1/\bar{f}_2$ vs. Δx is constant (compare Fig. 3 *a* and *c*). The corresponding phase difference vs. distance is given in Fig. 4. The measurements of the phase differences are performed perpendicular to the uniphase line (Machemer, 1974). This method is our routine technique from which the metachronal wavelength can be calculated (Eshel and Priel, 1987). It can also be regarded as a qualitative manifestation of ciliary synchronization in the given domain.

In order to test the generality of the behavior of synchronization between cilia, we have averaged the Pearson correlation factor over 30 different experiments. The examined tissue cultures were taken from 10 frogs which provided a body of data of reasonable size for averaging. As can be seen by comparing Figs. 3 *a* and 5, the Pearson factor drops with distance below 1 but stays very high (>0.8) even for relatively large distances. The distance was increased in steps of 0.5 μm and the measurements were performed over dimensions of one to two ciliary cells. The synchronization between beating cilia is decreased as the distance between measured cilia is increased. Two distinct stages can be observed: at relatively small separations ($\Delta x \leq 4$ μm) the synchronization factor decreases more strongly, whereas further increase in Δx causes relatively small decrease in synchronization. It seems that the synchronization factor approaches relatively fast a plateau value. In any case, it is possible to conclude that at relatively short distances the synchronization factor, between beating cilia is quite high ($\Delta x \leq 4$ μm, $\bar{\bar{P}} = 0.85$).

According to our definition the phase-corrected Pearson correlation factor should be independent of the direction of the measurement relative to the direction of the metachronal wave. To verify this, we performed measurements on the same ciliary cell in various directions relative to the metachronal wave (0°, 30°, 45°, 60°, and 90°); the same synchronization factor was observed in every case. As was already mentioned, our routine measurements are performed perpendicular to the uniphase line, in order to extract the metachronal wavelength as well. Therefore, the Pearson correlation factors are routinely measured in this direction.

Long-range synchronization

Even in tissue culture patches can be found on which the ciliary cells are organized such that they create a well-defined long-range metachronal wave. Such long-range organized domains, found by microscopic (×200) obser-

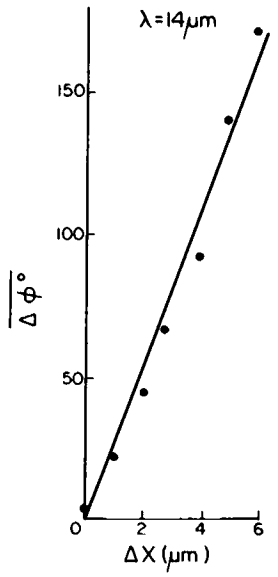

FIGURE 4 Phase difference vs. distance, the corresponding wavelength calculated from this graph is 14 μm.

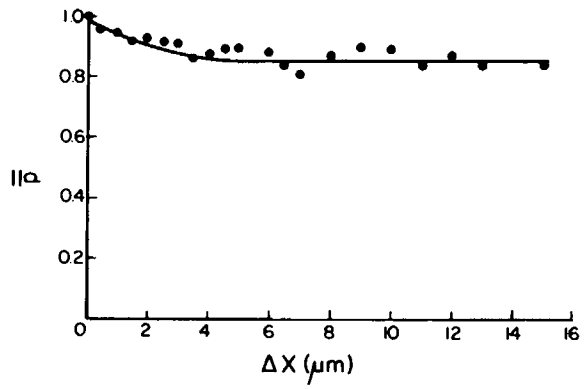

FIGURE 5 Average Pearson correlation factor vs. distance. The averaging is performed over 30 different cells. In addition, averaging over 40 s was also performed for every point.

vation, were chosen for this study. The typical behavior of synchronization factor, frequency, and $\bar{f}_1/\bar{f}_2$ vs. distance is shown in Fig. 6. The general appearance of the figure is similar to that of Fig. 3, which has already been explained in detail. We shall therefore limit ourselves here to emphasizing the salient features. The measurement was performed over 110 μm which comprises between 9 and 11 ciliary cells, assuming that their principal axes (~10 μm) are on a straight line. Even at such long distances relatively high synchronization (Δx = 110 μm, $\bar{P}$ = 0.67) between beating cilia exists (see Fig. 6 *a*). As a first approximation the average (over 40 s) Pearson correlation factor vs. distance was drawn as a smooth continuous line reaching a plateau value. There are strong indications that this is an oversimplified approach, and it seems that synchronization over long distances exhibits an oscillatory pattern with decreasing amplitude (Fig. 6). The length of such periodicity is between 30 and 50 μm. This phenomenon was observed in all long-range experiments performed by us. Such behavior of P vs. Δx can be explained in two different ways: (a) Along the measured line domains exist with higher or lower synchronization relative to the reference point. On top of that, P is a decreasing function of Δx; a linear combination of two such effects can reproduce the observed experimental curve. (b) The measurements at different Δx are performed also at different times (as was already explained).

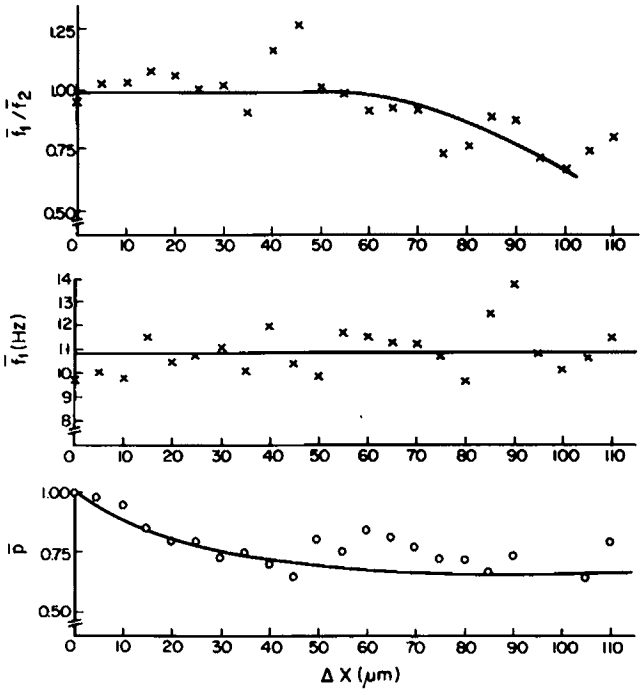

FIGURE 6 A typical long-range measurement (Δx = 110 μm), of the Pearson correlation factor, frequency ($\bar{f}_1$) and ($\bar{f}_1/\bar{f}_2$) as a function of Δx (similar to Fig. 3 for short-range measurements).

Over such long distances it takes a long time from the beginning of the experiment to its end. Measurements over distances of 50 μm (in steps of 5 μm) take half an hour. At such long times spontaneous fluctuations with higher synchronization may occur which will gradually decay to their normal values. It is evident that further work is needed, with the help of other techniques, to understand the mechanism that may cause such behavior.

The synchronization between average frequencies vs. distance as expressed by $\bar{f}_1/\bar{f}_2$, reveals relatively strong correlation over a distance of 40 μm. Further increase of distance shows a continuous decrease of synchronization between the average frequencies (Fig. 6 *c*). As was already explained deviations of $\bar{f}_1/\bar{f}_2$ from unity indicate lack of synchronization in frequency, while $\bar{f}_1/\bar{f}_2 = 1$ indicate complete synchronization.

Previously, we have described in detail the dependence of the phase difference on distance (Eshel and Priel, 1987; Ovadyahu and Priel, 1989). However, all of that work was concerned with relatively short distances ($\Delta x \leq 10$ μm) within the dimensions of a single ciliary cell, (similar to what is shown in Fig. 4). Moreover, as far as we know, with other techniques, the metachronal wave measurements were limited more or less to the dimensions on one cell. Fig. 7, however, represents phase difference vs. distance measured over 110 μm which is equivalent to about 10 ciliary cells. It is evident that, considering our degree of accuracy, it is impossible to draw a simple straight line between the experimental points. The

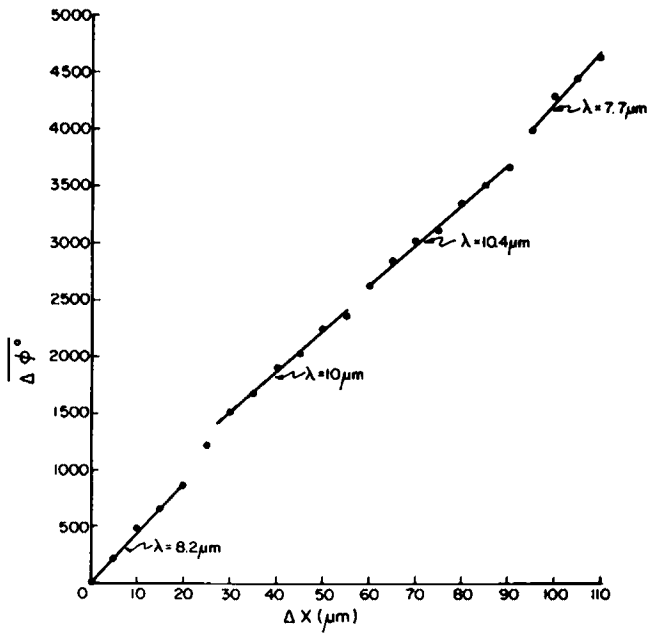

FIGURE 7 Phase differences vs. Δx over relatively long distances.

domain (110 μm) is divided into several sections 20–30 μm long, within each of which the experimental points fall on a straight line. These different lines are characterized by different slopes meaning different wavelengths as indicated in the figure. Although the differences in metachronal wavelength are only in the ragne of ±15%, they are definitely above our experimental error.

Synchronization between randomly chosen areas

Most of our effort was to measure the synchronization along the direction of the metachronal wave and in its vicinity. Correlation between cilia is essential to their physiological role of transporting materials. Hence it is logical to expect high values of synchronization as indeed was found (Figs. 5 and 6). However, in order to complete the description of synchronization between beating cilia, it is interesting to measure synchronization between ciliary beating on randomly chosen cells.

All of the randomly chosen pairs of areas were located on different patches, disconnected one from the other. The measurements reveals considerably smaller $\bar{P}$ values compared to those taken on the same patch (along the metachronal wave) at same distance apart. A typical behavior is presented in Fig. 8 *a*; as can be seen at $\Delta x = 10$ μm apart, two disconnected cells exhibit $P = 0.6$ which is lower than $\bar{P}$ values of connected cells at comparable separation distances (compare Fig. 5 to Fig. 8 *a*). Nevertheless, it is interesting to note that at small separation distances ($\Delta x \leq 10\mu$m) $\bar{P}$ values are relatively high even for disconnected cells, indicating relatively strong synchronization. Increasing Δx, $\bar{P}$ values decrease quite strongly and at $\Delta x = 25$ μm reach a "plateau" of relatively small $\bar{P}$ values. As can be seen in Fig. 8 *a*, at $\Delta x = 50$ μm $\bar{P} = 0.35$ and at $\Delta x = 110$ μm $\bar{P} = 0.33$, while $\bar{P}$ values at equivalent distances measured on one patch were larger by a factor of 2 (see Figs. 6 and 8 *a*). This plateau continues with small declining slope, and at the separation distances of 250 μm $\bar{P}$ is ~0.25. Our experimental system in its present version is capable of measuring, with relatively high accuracy, separation distances up to 270 μm.

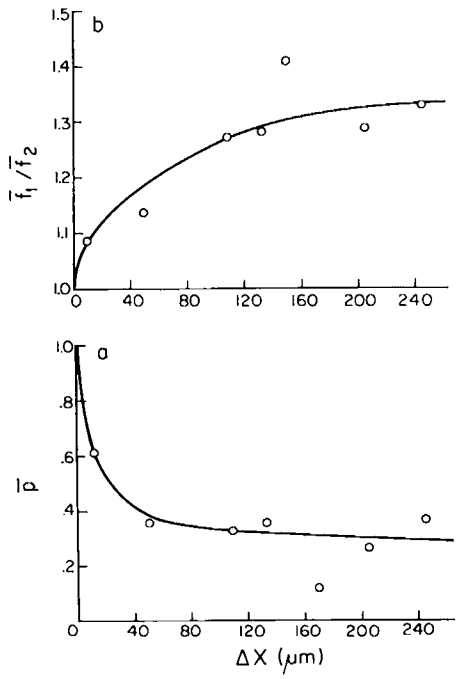

FIGURE 8 A typical Pearson correlation factor (*a*) and ratio of frequencies (*b*) as a function of Δx. The signals are derived from disconnected randomly chosen ciliary cells. Averaging was performed over 120 s for each point.

A first-order explanation to the strong decrease in $\bar{P}$ vs. Δx can be given by comparing frequencies from disconnected large distances apart pairs of areas. As can be seen, the discrepancy between the two frequencies increases with the distance of separation between the measured areas (Fig. 8 *b*). This means that the probability to find two disconnected ciliary areas that beat with similar frequencies decreases with the increase of Δx. However, the whole picture is far more complicated and changes in wave patterns were noticed between disconnected ciliary areas at large separation distances, as reflected by small $\bar{P}$ values.

The relatively strong synchronization that was observed even for disconnected ciliary cells, provided that they were close enough ($\bar{P} \geq 0.6$, $\Delta x \leq 10$ μm) is a very important finding. These results may shed light on the mechanism of ciliary metachronizm as will be discussed in detail below (see Discussion).

DISCUSSION

A measurable quantitative parameter that describes the degree of synchronization between beating cilia was introduced. The way in which we have defined this parameter (and having removed, as explained above, possible phase differences) describes the correlation between the wave patterns for the two areas under examination. The wave pattern reflects the collective behavior of the cilia in the measured area. The correlation factor was found to decrease monotonically with separation, Δx, starting with 1 at $\Delta x = 0$ and staying high, $\bar{P} > 0.8$ up to $\Delta x = 10$ μm (see Figs. 3 *a* and 4). Recently, it was shown (Eshel and Priel, 1987) that strong fluctuations in wave patterns exist even on a relatively short time scale (3 ms). If those fluctuations in ciliary beating are fully correlated between the measured two areas, then the Pearson corre-

lation factor should be 1. The decrease of $\bar{P}$ with Δx, even when the mean frequencies in both areas remain similar to one another, shows that these rapid fluctuations are only partially correlated. The $\bar{P}$ vs. Δx behavior (see Figs. 3, 4, and 6) seems to consist of a superposition of, at least, two processes: a rapid one which accounts for the initial loss of correlation over short distances (~10 μm) and a long-range one which preserves correlation over remarkably large separations preserving a value of $\bar{P} = 0.67$ at $\Delta x = 110$ μm.

An additional parameter, $\bar{f}_1/\bar{f}_2$, which, in principle, can also describe the synchronization between beating cilia was tested as well. In this method each of the 1-s spectra is represented by a single number which is its dominant frequency. With the Pearson correlation factor, on the other hand, we compare the two spectra point by point (in time). It is thus not surprising that $\bar{f}_1/\bar{f}_2$ will give a crude description of the degree of synchronization. It ignores additional frequencies with lower power and fast deviations or distortions which on this scale (1 s) are either small effects or are averaged out. Nevertheless, using this relatively insensitive parameter reveals that the range of synchronization between beating cilia has a value of 40–55 μm (Fig. 6). This length of relatively high synchronization is very similar to the effective length of ciliary stimulation (~50 μm) recently found by Spungin and Silberberg (1983). At this stage it is difficult to say whether these two findings are directly connected or whether the agreement is fortuitous.

The ciliary epithelium in the frog's palate, like most of the respiratory epithelium, is commonly regarded as a weak metachronal system. This work enables one to make such qualitative statements quantitative. We have shown that the synchronization is fairly high even over relatively large separations (Figs. 3 and 6) but it is definitely not perfect. Uncorrelated beating cilia does exist. In addition, even in well-organized ciliary patches it is impossible to describe the aligned cells by one metachronal wave, the wavelength being relatively short and different from one another (Fig. 7). The largest domains which can be characterized by a single metachronal wavelength are in the range of 20–30 μm. Two possible explanations to this findings are: (*a*) the principal axes of the ciliary cells in the patches are not perfectly aligned but rather rotated relatively to one another by some angle; or (b) slight ripples or folds in the tissue culture may exist. In either case, by measuring the phase difference vs. distance along some straight line in an average direction will introduce differences in the metachronal wavelength as was demonstrated for protozoa (Machemer, 1974) and by us for the tissue culture discussed here (Ovadyahu and Priel, 1989). Recently, it was shown that the ratio of particle velocity to the velocity of the metachronal wave is about 50% (Priel, 1987). This imperfect velocity coupling may be partially explained by the two findings of this work: the diminishing correlation over distance and the existence of different metachronal wavelengths even over one well-organized patch.

It was found that a relatively high correlation factor ($\bar{P} \geq 0.6$) exists between disconnected ciliary cells provided the gap between them is less than 10 μm wide. Therefore, synchronization can exist between beating cilia without direct contact between the cells. These findings agree with the early findings that when the heads of individual spermatozoa are close, their tails beat synchronously (Gray, 1930). To give another example, an organism, such as *Spirochaeta balbianii,* may attach itself to the side of a style by its end and continue to move the rest of its body in a lateral fashion. When a second such organism comes into the vicinity of the attached one it often also attaches itself. At first there is no coordination between their movements but within a few seconds complete coordination is established (Gray, 1930). Similar results were obtained by Okuna and Hiramoto (1976), regarding the mechanical modulation of the beating frequency of starfish sperm flagella. These authors were able to change the frequency of the flagella beating by applying rhythmic forces with frequency different from the intrinsic frequency of the flagellum, employing a microneedle. As far as we know there is no experimental evidence for such coupling or synchronization for separated dense ciliary epithelium. Here we show that similar phenomena occur also between disconnected ciliary epithelium cells. All the above mentioned findings can be explained by assuming that cilia are coupled to one another through the medium between them. Recently, a mathematical model involving an array of coupled oscillators was proposed. It showed (using mathematical analysis and computer simulation) the feasibility of self-synchronization and the formation of the metachronal wave in ciliary beating through hydrodynamical coupling of the cilia (manuscript submitted for publication).

To conclude, a sensitive parameter that characterizes the quantitative degree of synchronization between beating cilia was defined and applied. The degree of synchronization was found to be relatively high even over long distances when measured on a given patch. Moreover, synchronization exists between beating ciliary cells even when they are disconnected from one another provided the gap between them is within 10 μm. The average frequencies from two areas are correlated quite well to within 35–50 μm, after which a decrease in synchronization occurs. Even in carefully selected patches, in which the metachronism seems to be well defined as seen through a microscope, several metachronal wavelengths were found. Measurements of disconnected randomly chosen ciliary areas, separated by large distances ($\Delta x \geq 25$ μm) reveal a small synchronization factor, as

compared to $\bar{P}$ values for similar Δx when measured along the metachronal wave.

This research was supported by a grant from the Israel National Council of Research and Development.

Received for publication 14 March 1988 and in final form 7 September 1988.

REFERENCES

Aiello, E. 1974. Control of ciliary activity in Metazoa. *In* Cilia and Flagella. M. A. Sleigh, editor. Academic Press, Ltd., London. 353–376.

Aiello, E. and M. A. Sleigh. 1972. The metachronal wave of lateral cilia of *Mytilus edulis*. *J. Cell Biol.* 54:493–506.

Blalock, H. M. 1972. Social Statistics. 2nd edition. McGraw-Hill Book Co., Inc., New York.

Brenner, R. I. 1969. The biology of oviductal cilia. *In* The Mammalian Oviduct. E. Hafez and R. Blandau, editors. University of Chicago Press, Chicago, IL. 203–229.

Eshel, D., Y. Grossman, and Z. Priel. 1985. Spectral characterization of ciliary beating: variation of frequency with time *Am. J. Physiol.* 249:C160–C165.

Eshel, D., and Z. Priel. 1987. Characterization of metachronal wave of beating cilia on forg's palate epithelium in tissue culture. *J. Physiol. (Lond.)* 388:1–8.

Gorelik, G. S. 1959. Oscillations and Waves (in Russian). C. M. Ritov, editor. Physical and Mathematical Press, Moscow. 443–447.

Gosselin, R. E. 1966. Physiologic regulations of ciliary motion. *Annu. Rev. Respir.* Dis. 93:41–59.

Gray, J. 1928. Ciliary Movement. Cambridge University Press, London. 117–129.

Gray, J. 1930. The mechanism of ciliary movement. IV. Photographic and stroboscopic analysis of ciliary movement. *Proc. R. Soc. Lond. Biol. Sci.* 107:313–332.

Kennedy, S. R., and R. E. Duckett, 1981. The study of ciliary frequencies with an optical spectrum analysis system. *Exp. Cell Res.* 135:147–156.

Kinosita, H., and A. Murakami. 1967. Control of ciliary motion. *Physiol. Rev.* 47:53–82.

Machemer, H. 1974. Ciliary activity and metachronism in Protozoa. *In* Cilia and Flagella. M. A. Sleigh, editor. Academic Press, Ltd., London. 119–286.

Okuno, M., and Y. Hiramoto, 1976. Mechanical stimulation of starfish flagella. *J. Exp. Biol.* 65:401–413.

Ovadyahu, D., and Z. Priel, 1989. Characterization of metachronal wave in beating cilia: distribution of phases in spaces. *Biorheology*. In press.

Priel, Z. 1987. Direct measurement of the velocity of the metachronal wave in beating cilia. *Biorheology*. 24:599–604.

Seo, A. 1931. Studies in the nervous regulation of the ciliary movement *Jpn. F. Med. Sci. III Biophys.* 2:47–75.

Sleigh, M. A. 1962. The Biology of Cilia and Flagella. Pergamon Press, Oxford.

Spungin, B., and A. Silberberg, 1984. Stimulation of mucus secretion, ciliary activity, and transport in frog's palate epithelium. *Am. J. Physiol.* 247:C299–C308.

Zahm, S. M., J. Jaequot, and E. Puchelle, 1986. Ciliary beating frequency of frog palate and rat trachea explants under continuous perfusion. *Comp. Biochem. Physiol.* 85A:97–102.