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SPECTRAL CHARACTERIZATION OF CILIARY BEATING

BIOLOGICAL MEANING OF THE SPECTRAL LINEWIDTH

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The ciliary beating frequency in tissue culture from frog palate and lung was examined optically. Consecutive segments of the analog signal were then fast-Fourier transformed (FFT). The optical signals were measured as a function of the number of cilia by varying the examined area from 1.2 to 122 μm^2. The frequency of the maximum power of the spectra was independent of the measured area, while the line shape of the spectra and distribution of the main frequencies were strongly dependent on the dimensions of the area examined. The possibility that the width of the measured spectral peak reflects both the distribution of the beating frequencies and the distribution of the phases within the examined area is discussed.

1. Introduction

Recently the photoelectric method has been improved and adapted to measure the frequency of ciliary beating within small areas. Fast Fourier transformation (FFT) was routinely used for analyzing the signals obtained. These improvements in measuring and analyzing tools have permitted the determination of beating frequencies with higher accuracy [1–5]. Most of these experimental efforts concentrated on measuring the influence of chemicals, drugs and physical parameters on the frequency. However, it is well understood that the frequency alone cannot characterize the transport process of ciliary systems. Additional parameters are needed.

We intend to show here that besides the commonly measured parameter, the frequency, the FFT spectrum contains additional information, which is represented by the spectral linewidth and which has important physiological significance.

2. Materials and methods

2.1. Preparation

Experiments were performed on locally supplied frogs (*Rana ridibunda*). The animals were decapitated and the palate and lungs excised. The organs were washed and cleaned carefully with amphibian phosphate buffer solution (APBS) (Bio-Lab, Jerusalem), containing 100000 U penicillin, 100000 μg streptomycin and 10000 U mycostatin per 100 ml solution. The tissue was cut into small pieces (about 1 mm^2 each) which were each flattened on the bottom of a petri dish filled with a solution at pH 7.4, containing 65% L-15 medium (Bio-Lab), 15% fetal calf serum (Bio-Lab), 20% double-distilled water, and an antibiotic concentration of 1/10 of that in the APBS. The dishes were held under ultraviolet light, and the medium solution was replaced every 2 days. The incubation temperature was 23°C as was the tem-

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perature at which the measurements were made.

The measurements were taken from the third day until the 20th day, at which point the tissue in culture began to disconnect from the bottom of the dish. During the period of measurements, there was no obvious change in the ciliary activity. All the dishes, surgical equipment and materials were sterilized, and all operations on and treatment of the preparation were done in a sterile laminar-flow chamber (Haroshet, NB-4).

2.2. Optical measurement of ciliary beating and data analysis

The preparation was viewed using an inverted microscope (Olympus, IMT) with a 100 W tungsten-halogen lamp fed from a stabilized d.c. power supply (home-made). An optical fiber (50 μm diameter) was placed in the focal plane of the ocular (Gama Scientific, 700-10-36A) and connected to a photomultiplier (EMI, 9558B). The measurements were taken from the monolayer surface of the cultured epithelium which is transparent to light. The following objectives employed were: $\times 4$, $\times 10$, $\times 20$ and $\times 40$, which correspond to field diameters of 12.5, 5, 2.5 and 1.25 μm, respectively.

The photomultiplier output was further amplified (Par, 113A) and digitalized into a 32K memory unit of a home-made Z-80 microcomputer with a sampling rate of 170 Hz.

After each set of recordings, a control measurement of the system was taken by monitoring the signal from a nonciliary area of the tissue. The digitized signal was fed off-line into a CDC computer system for FFT and statistical analysis. The data acquisition system and the computer analysis were calibrated with three light sources oscillating simultaneously either at three different frequencies, or at the same frequency. The calibrations were made at different objective magnifications. The output spectra revealed the input frequencies only, regardless of the objective magnification.

3. Results

Our experimental setup permits measurement of considerably small subcellular regions, ranging from 1.2 to 122 μm^2, simply by varying the magnification of the microscope objective. The data collected from field diameters of 12.5, 5, 2.5 and 1.25 μm reflect the beating of 80, 13, 3 and 1 cilia, respectively, assuming that on a single ciliary cell (~ 75 μm^2) there are about 50 cilia [6], and the ciliary density is uniform.

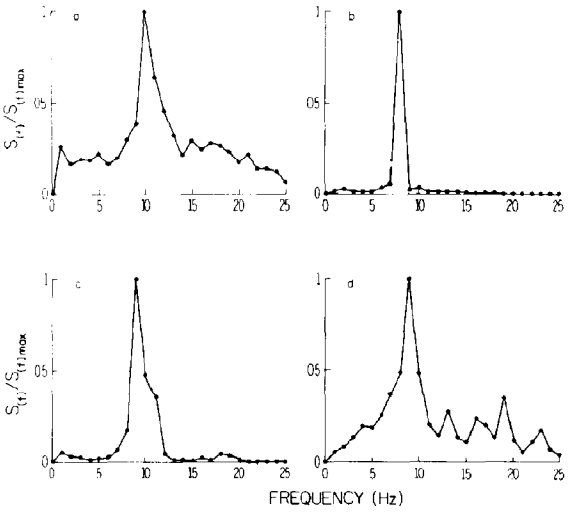

Fig. 1. 10-s normalized FFT spectra. These spectra were observed from areas with field diameters of (a) 1.25, (b) 2.5, (c) 5 and (d) 12.5 μm.

Since there was no doubt that the effects measured are statistical in nature with quite a wide dispersion, relatively large samples were taken. A set of experiments, including at least 100 spectra for every magnification, was performed on the same day. The experiments continued for more than 2 weeks on the same culture with no chronological time effect observed. The same experiment performed on 10 different frogs gave the same basic results.

Typical spectra of 10-s measured signals (observed from the same location at different times) at four different field diameters are shown in fig. 1. As can be seen, the spectral linewidth becomes considerably narrower when increasing the field diameter from 1.25 μm (fig. 1a) to 2.5 μm (fig. 1b). Further increasing the field diameter to 5 μm (fig. 1c) and then to 12.5 μm (fig. 1d), resulted in a broadening of the spectral linewidth.

We collected the frequencies with maximum

power from 200 10-s spectra of each magnification. The distributions of these frequencies are presented in fig. 2. These distributions become narrower when increasing the field diameter from 1.25 μm (fig. 2a) to 2.5 μm (fig. 2b) and then to 5 μm (fig. 2c). However, when we increased the diameter to 12.5 μm, the distribution became wide again (fig. 2d).

The line shape of the spectra and of the distribution of frequencies depends on the size of the observed area as can be seen from the figures represented here. However, in order to analyze this mathematically, we calculated the expectation value ($\bar{f}$) defined as

$$\bar{f} = \frac{\sum s_i f_i}{\sum s_i} \tag{1}$$

and the S.D. (standard deviation), given as:

$$\text{S.D.} = \sqrt{\frac{\sum s_i (f_i - \bar{f})^2}{\sum s_i}} \tag{2}$$

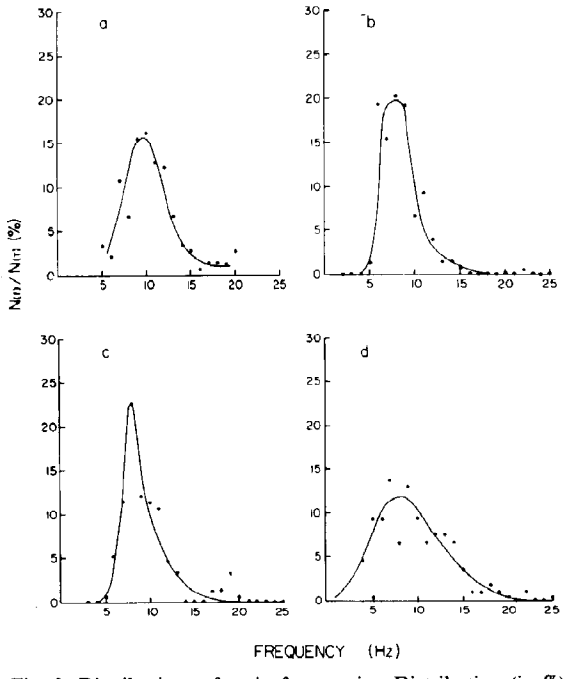

Fig. 2. Distributions of main frequencies. Distribution (in %) of the maximum power frequencies, taken from 10-s spectra at the same field diameters as in fig. 1.

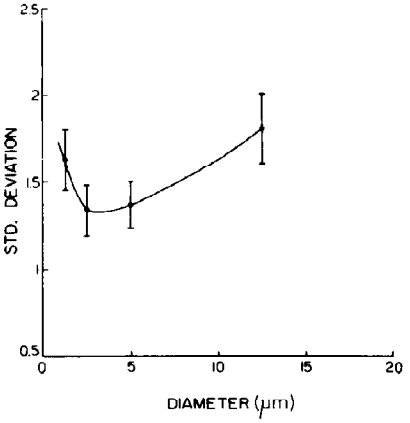

Fig. 3. S.D. as a function of field diameter. Average S.D. (eq. 2) as a function of the diameter of the viewed area. Every point on this curve represents an average of 200 values.

where f_i is the i-th frequency and s_i its power.

Averages of the expectation values (eq. 1) and the S.D. (eq. 2) were calculated from 200 10-s spectra at each field diameter. It was found, within the accuracy of our measurements, that the expectation value did not depend on the number of cilia measured, while the S.D. did (fig. 3). When the system was calibrated with oscillating lamps, the expectation value and the S.D. were independent of the objective magnification. Such calibration shows that our experimental setup and data analysis can differentiate between the existing frequencies, whether close to or far away from each other, without any noticeable distortion. It therefore seems highly unlikely that our findings are artifacts of the measurement or analysis setup.

On all measured S.D. at the four different magnifications we performed a test of all possible pairs of group means (Duncan experiment) [7]. The level of significance was set at $p = 0.001$. It was found that at this high accuracy there is no difference between the means of S.D. at the 2.5 and 5 μm diameters. However, the S.D. means for the 1.2 and 12.5 μm diameters were different from the 2.5 and 5 μm values and from each other.

Based on the above it is apparent that the S.D. is dependent on the size of the observed field. All the above results were obtained from frog palate culture. Similar results were observed with frog lung cultures.

4. Discussion

In order to explain our experimental findings we suggest the following model. For small areas of frog palate ciliary epithelium, cilia beat with a metachronal pattern as was shown for short periods of time by Aiello and Sleigh [6]. However, it was shown [5] that even within the metachronal wave, a fraction of the cilia might beat temporarily at frequencies different from the main frequency at which most of the cilia beat. It is reasonable to assume that these cilia which beat at different frequencies will be forced in time to beat at the main frequency of the metachronal wave, according to the principle of 'minimum interference' [8]. While the cilia having beaten at a frequency different from the main frequency revert to the latter other cilia 'stray' and start beating at a different frequency. It is a dynamic process. Therefore, when we measure the relatively small number of cilia (one to two), such fluctuations should be strongly reflected by the width of the spectral peak.

According to the ergodic hypothesis [9], the width of the spectral peak should become narrower as we increase the periods of time being analyzed, as was demonstrated previously [5]. Extending the time of analysis is equivalent to increasing the number of cilia measured which in principle should narrow the width of the spectral peak. If the fluctuations in frequency are about the same average value, then the width of the spectral peak will become narrower by a factor of $1/\sqrt{N-1}$, where N is the number of cilia measured. This is indeed observed in fig. 3, where the S.D. value for one cilium is 1.71. Increasing the number of measured cilia to three should narrow the spectral width to 1.21 as compared to the measured value of 1.34. The difference between measured and calculated values is within our experimental error. However, by further increasing the number of cilia to 15 the peak width becomes larger contrary to the expectations of the ergodic hypothesis. This behaviour can be explained by introducing a heterogeneity into the system, in addition to the fluctuation in frequency.

One possible explanation might be, that even on the same cell, there exist groups of cilia which beat around different main frequencies. We have checked this possibility by measuring the main frequency from various places within the same cell, and found that, within our experimental error, the main frequency remains unchanged within a circle width of about 5 μm diameter. When measuring the main frequency outside such a circle, especially when, during scanning, we pass into another cell, we occasionally find groups of cilia which beat around different frequencies. Therefore, this heterogeneity cannot explain the broader peak at the 5 μm field diameter measurements. This is a possible explanation for the very broad peak at the 12.5 μm field diameter, which covers more than a single ciliary cell. Another possibility is that a distribution of phases results simply due to the fact that the metachronal wave is seen with different phases at different locations inside the examined area. Such heterogeneity in phases, should broaden the spectral peak as we will now explain. Let us assume that a cilium beating with a constant frequency, ω_0, produces a periodical optical signal, which is represented by $g(t)$. The value of the Fourier transform at ω_0 is $G(\omega = \omega_0)$. It can be shown that when we have another cilium which beats with the same frequency, ω_0, but with a phase difference, φ, and produces a signal represented by $g(t,\varphi)$, its Fourier transform value at ω_0 will be $e^{-i\varphi}G(\omega = \omega_0)$. Thus, partially destructive interference may occur between cilia beating at the same main frequency but not in phase.

According to our model the signal may be visualized as being composed of two contributions: the signal produced by cilia beating with the metachronal wave frequency ω_0 and that produced by cilia temporarily 'out of tune' and beating with various frequencies around ω_0. The cilia beating with ω_0 are phase correlated whereas the others are not. However, taking a field of finite size means observing simultaneously ω_0 with different phases corresponding to their different locations in the field. This produces a distribution of phases leading, as explained above, to a lowering of intensity at ω_0, the center of signal. No comparable effect occurs at the wings, at the various frequencies around ω_0, since the out of tune cilia are not phase correlated to begin with. The net result, therefore, is a lowering of the center of

the peak, relative to the wings, an effect which appears as signal broadening.

We have shown that a phase distribution within the cilia which beat at the main frequency results in a broader peak at the FFT spectrum. Aiello and Sleigh [6] found that the length of the metachronal wave at the frog palate epithelium is 10–15 μm. According to this finding we might have a phase difference at 180° between the cilia which are located at opposite edges of the 5 μm diameter circle, which will certainly contribute to widening of the peak.

Additional support for the suggested model is provided by the observation that when we present the distributions of the main frequencies of the spectra at different field diameters, the system behaves in an ergodic manner even with a field diameter of up to 5 μm (fig. 2). This is because we take into account only the frequency of maximum power for each spectrum, ignoring the width of the peaks and thus the phase distribution.

Puchelle et al. [3] recently noted that the number of cilia measured does not influence the spectra observed. The main difference between their experiments and ours was the range of the areas chosen. Our upper limit (122 μm^2) was the same as their lower one. Therefore, these two results may not be contradictory.Moreover, their results could be explained according to our suggested model which predicts that by increasing either the time of observation or the number of the cilia measured the effects will be averaged into a relatively broad spectrum. In the present work we have been able to monitor small areas for short periods of time which has allowed us to probe this system in greater detail than was previously possible.

It is well known that the frequency alone cannot characterize the ciliary beating process. Here we present an additional parameter, the S.D. (eq. 2), which represents the distribution of frequencies and/or phases within the measured area. However, by choosing the appropriate experimental conditions these two effects can be separated. Changes in frequency fluctuations due to imposed experimental conditions on ciliary tissue can be estimated by examining relatively small areas (~ 1 μm^2) where one to two cilia are measured. Under these conditions either distribution of phases does not exist (one cilia) or is so small that it can be ignored (two cilia). Therefore, relative changes in S.D. represent solely changes in frequency fluctuations. If areas larger than 5 μm^2 are examined the measured S.D. represents simultaneously frequency fluctuations and light cross-correlations due to space phase distribution created by the metachronal wave. Even under such experimental conditions, by careful examination of the distribution of frequencies of spectral maxima and the S.D. values of the different areas observed, we can differentiate between the two mentioned effects.

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