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Analysis of chemotaxis when the fraction of responsive cells is small - application to mammalian sperm guidance

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ABSTRACT The detection of chemotaxis-related changes in the swimming behavior of mammalian spermatozoa in a spatial chemoattractant gradient has hitherto been an intractable problem. The difficulty is that the fraction of responsive cells in the sperm population is very small and that the large majority of the cells, though non-responsive, are motile too. Assessment of the chemotactic effects in a spatial gradient is also very sensitive to the quality of sperm tracking. To overcome these difficulties we propose a new approach, based on the analysis of the distribution of instantaneous directionality angles made by spermatozoa in a spatial gradient versus a no-gradient control. Although the use of this parameter does not allow identification of individual responding cells, it is a reliable measure of directionality, independent of errors in cell tracking caused by cell collisions, track crossings, and track splitting. The analysis identifies bias in the swimming direction of a population relative to the gradient direction. It involves statistical χ^2 tests of the very large sample of measured angles, where the critical χ^2 values are adjusted to the sample size by the bootstrapping procedure. The combination of the newly measured parameter and the special analysis provides a highly sensitive method for the detection of a chemotactic response, even a very small one.

KEY WORDS: *sperm chemotaxis, chemotaxis analysis, chemotaxis assay, chi square test, bootstrapping*

Introduction

In most systems in which chemotaxis has been studied (e.g., in bacteria, in cells and species with amoeboid movement, and in sperm cells of marine invertebrates), a large fraction of the cells, often nearly all of them, apparently respond to the chemoattractant gradient (Eisenbach, 2004a). In the case of the more recently found system of mammalian sperm chemotaxis, the situation is different. There only a small fraction of the cells [2–12% in humans (Cohen-Dayag *et al.*, 1994, Spehr *et al.*, 2006)] are chemotactically responsive (Eisenbach and Giojalas, 2006). This fractional response poses a technical difficulty: how can one measure the small fraction of reacting spermatozoa in the presence of ~10-50 fold larger number of normally swimming, yet non-responsive, spermatozoa? The same question applies to mammalian sperm thermotaxis, where the fraction of responsive cells is similarly low (Bahat *et al.*, 2003), because in both chemotaxis and thermotaxis only the small fraction of capacitated spermatozoa is responsive (Eisenbach and Giojalas, 2006). This paper deals with chemotaxis of human spermatozoa, but it equally well applies to any type of taxis and to any population containing cells that actively swim up or down a gradient of a stimulus.

Although the simplest and most popular assay for measuring chemotaxis is based on sperm accumulation, by itself it is not a reliable assay because processes other than chemotaxis can cause accumulation as well (Eisenbach, 1999, Eisenbach, 2004a). Unequivocal assays for chemotaxis (and other taxes) are based on the directionality of swimming. In the case of chemotaxis, responsive cells are expected to move in the direction of the chemical gradient, while non-responsive spermatozoa move in random directions. The first directionality assays with human spermatozoa involved manual drawing of tracks made by swimming spermatozoa near a chemoattractant source, and subsequently counting those with directional changes toward this source (Jaiswal *et al.*, 1999, Ralt *et al.*, 1994). As such, these assays were subjective and qualitative. The next generation of directionality assays employed objective, computerized analysis of the tracks made by the spermatozoa (Fabro *et al.*, 2002, Spehr *et al.*, 2004). Although this type of assay is specific for chemotaxis (and other taxes), the signal-to-noise ratio is very low when the fraction

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of responsive cells is small as in the case of human spermatozoa. [This is also true of other chemotaxis assays, including accumulation assays (Eisenbach, 1999, Ralt *et al.*, 1994).] The outcome of this, combined with the fact that these analyses only provide the average behavior of the sampled cells is that the changes in the measured parameters are very small, occasionally smaller than the noise (Bahat *et al.*, 2003, Fabro *et al.*, 2002, Sun *et al.*, 2005, Sun *et al.*, 2003). Furthermore, analyses based on tracks of moving cells are very sensitive to the quality of sperm tracking, and reduced quality may well lead to tracking errors. The tracks are especially error prone when cells swim close to each other. Such errors might well change the outcome of the analysis when the measured change is very small. The aim of this study was to find a way to reliably analyze chemotaxis in such cases. We propose a new approach for the evaluation of chemotaxis on the basis of statistical analysis of the instantaneous directionality angles that constitute the tracks.

Results and Discussion

Track-based analysis of directionality

All the parameters that have been proposed for track-based evaluation of chemotaxis in mammalian spermatozoa take into account only the first and last tracking points. These parameters include the average displacement of a population along the gradient direction ΔX (Fabro *et al.,* 2002), the angle of track direction relative to the gradient direction averaged over the sperm population (Spehr *et al.,* 2003), and, similarly, the percentage of cells having this angle in the range of ±45° (the percentage of cells with $\Delta x/|\Delta y|>1$) (Fabro *et al.,* 2002). These parameters provide the average behavior of the population or the percentage of cells that satisfy a certain criterion. Therefore, they are good for a substantial response, but their fidelity is much reduced when the percentage of responsive cells is low. In such a case, the correctness of cell tracking is crucial. Modern tracking software usually allows relatively sensitive tuning in order to take into account variations in cell morphology and acquisition conditions. However, none of the available motion analysis systems is error-free. The most common errors are caused by cells that pass very close to each other and their tracks are exchanged, forming trajectories composed of segments from two different cells, or by difficulties in cell recognition, resulting in splitting tracks into two or more fragments. In our hands, using a sophisticated motion analysis system, these erroneous tracks can amount to as high as 10% of the tracks.

Our first attempts to overcome these difficulties were at two levels. In one, we tried to find a parameter that would provide more robust results, i.e., better discrimination between random and biased movement. An example is the fraction of time spent in moving along the chemoattractant gradient averaged over the cell population, thus relying on the whole track rather than on the initial and final cell locations only. In the other, we developed tracking software that enables us to compare the

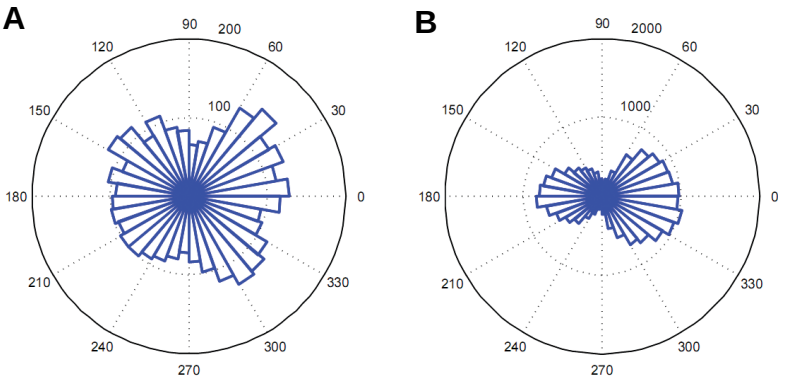

Fig. 1. Circular distribution diagrams of the instantaneous directionality angle (γ_{inst}) for *E. coli.* *The circular axis corresponds to the angle relative to the gradient direction. The radial axis corresponds to the number of angles. The diagrams are for the movement of* E. coli *cells within 1 min on the bridge of a Zigmond chamber, 5 min after sealing the chamber. See Materials and Methods for details.* **(A)** *Negative control (no chemoattractant gradient).* **(B)** *Chemoattractant gradient (its direction defined as 0° or the X axis).*

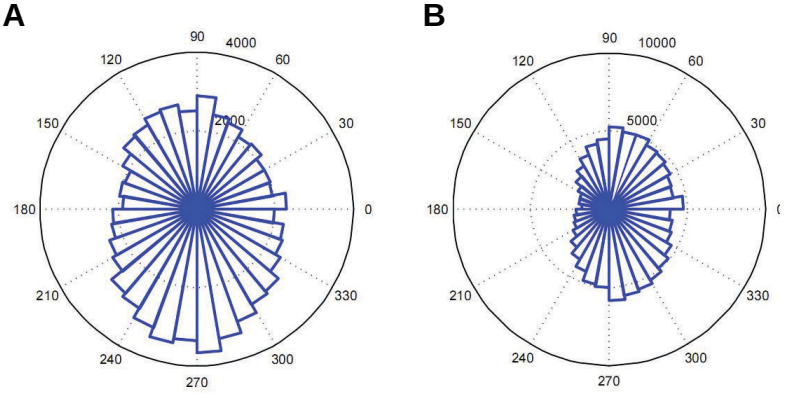

Fig. 2. Circular distribution diagrams of γ_{inst} for rabbit spermatozoa. (A) *Negative control (no temperature gradient).* **(B)** *Temperature gradient of 10°C/cm (37 to 39°C over 2 mm) along the X axis. The γ_{inst} values were calculated from the data of Bahat* et al. *(2003).*

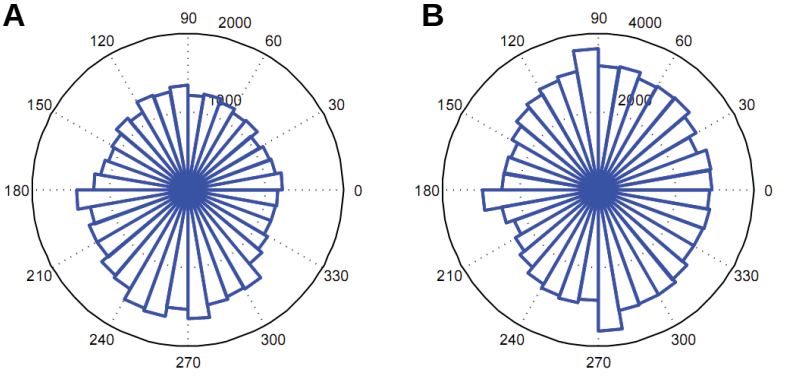

Fig. 3. Circular distribution diagrams of γ_{inst} for human spermatozoa. (A) *Negative control (no chemoattractant gradient).* **(B)** *Gradient of a cumulus-conditioned medium, serving as a chemoattractant (Sun* et al., *2005). The γ_{inst} values were calculated from the data of Oren-Benaroya* et al. *(submitted).*

TABLE 1

RESULTS OF RAYLEIGH TEST FOR UNIFORM DIRECTIONALITY

	Control					Taxis				
	N	θ	r_{mean}	***z***	***P***	***N***	θ	r_{mean}	***z***	***P***
E. coli chemotaxis	3,690	6°	0.05	10.5	<0.001	20,922	356°	0.13	361	<0.001
Rabbit sperm thermotaxis	90,642	265°	0.08	517	<0.001	143,583	347°	0.16	3,436	<0.05
Human sperm chemotaxis	46,326	250°	0.06	193	<0.001	103,832	12°	0.04	145	<0.001

The *P* value indicates the significance of the Rayleigh z-test (Zar, 1999).

true track of the cell with the one constructed by the motion-analysis system and, in the case of a difference, to correct the erroneous track. However, these measures were of no help: the new parameters yielded no better results, and the track fixing, though producing reliable cell tracks, turned out to be impractical due to the large number of tracks that had to be examined for correctness and the long time that it took to check and, if necessary, fix each track.

Track-independent analysis of directionality

The strong impact of the tracking quality on the fidelity of the assessment of a small directionality bias requires that the 'ideal' chemotactic parameter would be independent of the tracking quality. This requirement can be fulfilled by considering a set of instantaneous parameters of the movement instead of the same parameter measured as a single value for the whole track. The choice of the parameter depends on a priori knowledge of the features of movement in chemotaxis. Thus, for leukocyte chemotaxis, which is tightly linked to chemokinesis, the parameter used was the instantaneous displacement or the instantaneous velocity (Debeir *et al.,* 2004). For chemotaxis of ascidian spermatozoa, the parameter is the instantaneous distance between the cell and the chemoattractant source (Yoshida *et al.,* 2002). We did not use any of these parameters because both of them depend on the speed of swimming whereas, for human spermatozoa, we needed to have a directionality-based parameter. Instead, we considered using the instantaneous directionality angle, γ_{inst}, the angle between the vector of the cell frame-to-frame displacement and the gradient direction. Our expectation was that in the case of random movement the angles would be uniformly distributed in all directions, and that in the case of taxis of any kind the distribution would be biased toward the gradient direction. To verify this expectation, we chose two systems with a clear tactic response: chemotaxis of *Escherichia coli* cells [the best-characterized chemotaxis system, where most of the cells are chemotactically responsive (Eisenbach, 2004b)] and thermotaxis of rabbit spermatozoa [where the response, restricted to the fraction of capacitated cells, is clearly detectable by the conventional track-based parameters (Bahat *et al.,* 2003)]. Next we measured the distribution of γ_{inst} in the case of human sperm chemotaxis.

Analysis of bacterial chemotaxis

In the absence of stimuli, *E. coli* cells execute a random walk composed of runs and tumbles with essentially no net vectorial movement. In a chemoattractant gradient, the random walk is biased in the direction of the gradient: runs up the gradient are prolonged, and runs down the gradient are shorter (Macnab and Koshland, 1972). We studied the response of *E. coli* in the same chemotaxis chamber as was used for the measurement of sperm chemotaxis. We employed a potent chemoattractant mixture, consisting of L-serine (100 μM) and L-aspartate (1 μM) (Mesibov and Adler, 1972). We plotted the measured γ_{inst} values as a circular histogram for the whole sample. The presence of the chemoattractant gradient clearly biased the cell movement in the direction of the gradient, reflected in a very pronounced asymmetry of the circular distribution diagram of γ_{inst} (Fig. 1).

Analysis of sperm thermotaxis

We examined the γ_{inst} distribution for thermotaxis of rabbit spermatozoa. The circular histogram of γ_{inst} values clearly showed a bias along the Y axis (towards 90 and 270º) independently of whether or not a temperature gradient was established along the X axis (Fig. 2). This gradient-independent bias was observed in the Zigmond chamber with all sperm samples from both rabbit (Fig. 2) and human (Fig. 3) sources. The cause of this bias is not known. However, on top of it, a bias in the direction of the gradient (the X axis) was clearly observed (Fig. 2B).

Analysis of sperm chemotaxis

From data of human sperm chemotaxis, we observed asymmetry along the X axis in the direction of the chemoattractant gradient, on top of the gradient-independent bias along the Y axis (Fig. 3). To quantify the bias in the chemoattractant gradient direction, we used two approaches: circular statistics to identify grouping of angles, and a newly developed method to compare the asymmetries in the γ_{inst} distribution between the chemoattractant presence and absence.

Assessment of chemotaxis on the basis of γ_{inst} distribution

Methods of circular statistics

We first tried circular statistics - a commonly used branch of statistical methodology that focuses on distribution of directional parameters. First, for each sample of *N* measured values of γ_{inst}, we calculated two parameters of the mean vector: its direction, θ,

TABLE 2

RESULTS OF THE χ^2 TEST FOR ASYMMETRY OF ANGULAR DISTRIBUTION

	Control		Taxis		Odds Ratio	χ^2 test	
	N	**Odds**	***N***	**Odds**		χ^2	***P***
E. coli chemotaxis	1,943	1.16	14,259	1.39	1.2	13.5	$2{\cdot}10^{-4}$
Rabbit sperm thermotaxis	39,268	1.00	59,119	1.93	1.93	2,476	$<1{\cdot}10^{-4}$
Human sperm chemotaxis	21,497	0.93	48,059	1.15	1.21	187	$<1{\cdot}10^{-4}$

N (= $N_+ + N_-$) indicates the total number of angles in two bins, centered on 0 and ±180°. *P* indicates the significance of the χ^2 test.

according to

$$\theta = \arctan(S/C) \quad (1)$$

where $S = 1/N \cdot \sum \sin\gamma_{inst_i}$; $C = 1/N \cdot \sum \cos\gamma_{inst_i}$ and its length, r_{mean} which may vary from 0 (random distribution of angles) to 1 (all angles at the same direction), according to

$$r_{mean} = (C^2 + S^2)^{1/2}. \quad (2)$$

Then we applied the Rayleigh *z*-test for uniform directionality around the circle (Zar, 1999),

$$z = N \cdot r_{mean}^{\ 2}, \quad (3)$$

where high r_{mean} and *z* values indicate a tendency of γ_{inst} to cluster around θ. The data for the three cases of taxis mentioned above are shown in Table 1. In all of them, the mean direction θ was close to the gradient direction. As expected, in the cases of bacterial chemotaxis and rabbit sperm thermotaxis the r_{mean} values were higher than those of the respective controls. In contrast, in the case of human spermatozoa, the r_{mean} value in a gradient appeared to be similar to the no-gradient control, suggesting no chemotaxis. Paradoxically, however, the z values were significantly higher than the threshold values for all three cases both in the presence and absence of a gradient (Table 1), which raises the question as to whether this analysis is adequate for this type of experiment. There are two main reasons why the analysis may not be adequate: (a) Deviation from uniformity apparently cannot serve as a measure of the tactic response because in all cases, including the controls, the Rayleigh *z*-test showed extremely significant non-uniformity of the angular distributions. This was probably due to the linear correlation of the *z*-statistic with the number of measured angles (Eq. 3), which was very high. (b) We lack a statistical measure for the differences in r_{mean} between the presence and absence of a gradient within a single experiment.

TABLE 3

CRITICAL χ^2 VALUES TABULATED IN ORDER TO EVALUATE THE CHEMOTACTIC EFFECT IN A ZIGMOND CHAMBER

	α (significance level)					
	0.001	0.005	0.01	0.025	0.05	0.10
Set size	*New critical χ^2 values*					
5,000	24.1	19.3	17.2	14.9	12.4	9.9
10,000	31.1	26.0	23.6	20.6	17.8	14.9
15,000	38.0	32.7	30.0	26.3	23.2	20.0
20,000	45.0	39.5	36.4	31.9	28.6	25.0
	Regular critical χ^2 for df = 1 (*)					
Not specified	10.83	7.879	6.635	5.024	3.84	2.706

(*) The regular critical χ^2 values for the same significance levels and 1 df are shown for comparison.

TABLE 4

COMPARISON BETWEEN THE NOVEL AND CONVENTIONAL MODES OF ANALYSIS

No. of experiments yielding statistically significant positive results in both methods	5
No. of experiments yielding statistically significant positive results in the novel method and negative results in the conventional method (including results that appeared positive but were found statistically insignificant in the latter)	13
No. of experiments yielding negative results in both methods	3
No. of experiments yielding negative results in the novel method and statistically significant positive results in the conventional method	0

Asymmetry of angular distribution and the Odds Ratio parameter

The distinctive feature of circular distribution diagrams is asymmetry in the direction of the gradient. The degree of asymmetry can be simply assessed by the ratio between numbers of angles in two directions only: 'up' and 'down' the gradient. Because the small values of r_{mean} discussed above, result in part from imperfections in the angle distribution in irrelevant directions, we tried to reduce this noise by considering only angles within the range ±45° up or down the gradient. Therefore, we defined two bins, each 90° wide, centered at 0 and ±180° (the direction of the chemoattractant gradient being defined as 0°). Having the total numbers of angles in the 0 and 180° bins, N_+ and N_- respectively, we evaluated the degree of asymmetry of the circular distribution diagram by the Odds parameter, where Odds = N_+/N_-. The Odds parameter yields values close to 1 when the swimming is random; it is >1 when the swimming is biased in the gradient direction. The significance of the difference between the two groups of data can be estimated by the χ^2-test. The strength of the tactic response can be obtained by the Odds Ratio parameter (Odds Ratio = $Odds_{treatment}/Odds_{control}$). When we applied this analysis to the three systems of taxis, dealt with above, we observed a highly significant tactic response in each of these systems (Table 2).

The high sensitivity of the Odds Ratio to asymmetry of the angular distribution seemed to make it a good method for identifying even a small bias of movement in a specific direction. However, this method had the drawback that although the χ^2 test used in the data analysis may have yielded highly significant values, this could be merely due to the large sample size. Indeed, the large number of measured γ_{inst} values often caused two control replicates within the same experiment to be statistically different. The reason for this drawback lies in the fact that the critical χ^2 value found in statistical tables is determined according to the degrees of freedom (df) by the dimension of the table, which in our case is 2x2. Hence the χ^2 test has 1 df, regardless of the sample size. On the other hand, the χ^2 test statistic tends to increase as the sample size increases, due to the fact that it is based on a sum of non-negative components. Therefore, the more values added, the bigger the sum. To resolve this drawback we had two options: either to decrease the number of measurements, or to adjust the χ^2 critical value to large sample sizes. The first option seemed undesirable because it required a decrease in the number of monitored cells, just the opposite of what is needed when a response is small relative to the background. We therefore chose the second option, looking for a way to adjust the χ^2 critical values to the relevant sample sizes. The method developed is described in the Appendix. The outcome was a table of new empirical critical χ^2 values (Table 3). We employed this table to evaluate the significance of the observed asymme-

try, and hence whether or not a chemotactic response occurred.

Comparison between the novel and the conventional modes of analysis

To determine whether the new method, involving the measurement of γ_{inst} and the assessment of the bias by the χ^2 test with adjusted critical values, has indeed more power than the conventional analysis, we compared it with the conventional track-based analysis. We made the comparison for 21 chemotaxis assays carried out with human spermatozoa, employing progesterone (10 pM) as a chemoattractant (Teves *et al.,* 2006). As shown in Table 4, the new method of analysis improved to a large extent the outcome of the chemotaxis assays, and in no case did it yield false negative results. We wish to emphasize that the only effect of the new method is to increases the sensitivity of the analysis; this method does not amplify non-existing signals.

Conclusion

This study provides a new approach to the analysis of chemotactic responses. It involves the measurement of γ_{inst} values (instead of long tracks), determination of the bias of their distribution in the gradient direction, and evaluation of this bias relative to a no-gradient control by a χ^2 test adjusted to the sample size. This combined approach provides a highly sensitive method for the detection of a chemotactic response, even a hard-to-detect one. The disadvantage of this approach is that it does not allow the identification of individual responding cells needed for the assessment of the fraction of responsive cells. The sensitivity of the proposed method was demonstrated on human sperm chemotaxis but it can, in principle, be applied to any type of taxis and to any population of cells that actively move up or down a gradient of a stimulus.

Materials and methods

Spermatozoa

Human semen samples were obtained from two healthy donors after 3 days of sexual abstinence. Informed consent was obtained from each donor. Semen samples with normal sperm density, motility and morphology [according to WHO guidelines (World Health Organization, 1993)] were allowed to liquefy for 30–60 min at room temperature. Human spermatozoa were separated from the seminal plasma by the migration–sedimentation technique (Hauser *et al.,* 1992). Following this procedure, the sperm concentration was adjusted to $2x10^6$ cells/ml. The sperm suspensions were incubated under an atmosphere of 5% CO_2 at 37ºC for 2 h to obtain capacitated spermatozoa (Cohen-Dayag *et al.,* 1995). The capacitating medium, BWW, was essentially Biggers, Whitten and Whittingham medium — 95 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 20 mM sodium lactate, 5 mM glucose, and 0.25 mM sodium pyruvate, pH 7.4 (Biggers *et al.,* 1971), supplemented with HEPES (50 mM, pH 7.4) and 0.3% human serum albumin (Irvine Scientific, Santa Ana, CA, USA).

Bacteria

E. coli RP437 strain, wild type for chemotaxis (Parkinson and Houts, 1982), was grown overnight at 35°C in Luria Broth. The culture was diluted 100-fold in the tryptone broth and was grown to OD_{590} = 0.5–0.6 at 35°C. Cells were washed twice in motility buffer [KP_i (10 mM; pH 7.0), EDTA (0.1 mM), and methionine (0.1 mM)] and then resuspended in it.

Attractants

For the measurement of sperm chemotaxis, progesterone (10 pM) was used as a chemoattractant. The mixture solution of 100 μM L-serine and 1 μM of L-aspartate was used as the chemoattractant for bacteria.

Chemotaxis assays

Chemotaxis assays were performed, as described earlier (Fabro *et al.,* 2002), at 37°C in a Zigmond chemotaxis chamber consisting of two parallel, rectangular wells separated by a wall and closed with a coverslip (Zigmond, 1977). Unless indicated otherwise, one well contained cells in buffer [BWW for spermatozoa, motility buffer for bacteria] and the other contained chemoattractant solution or, as a control, buffer only. Following sealing of the chamber, the cells in it were allowed to equilibrate for 10 min, and then the movement of cells on top of the partition wall, in the middle of the field between the two wells, was video-recorded for 5 min at 10X under a phase-contrast microscope (spermatozoa) or at 40X under a dark-field microscope (bacteria). The cell tracks were subsequently analyzed with homemade software (written in Matlab).

Thermotaxis assays

Thermotaxis assays were performed as described earlier (Bahat *et al.,* 2003) in a modified Zigmond chamber, so that the temperature in each well could be accurately controlled and measured (±0.2°C). Rabbit spermatozoa at equal concentrations were put in both wells and the tracks of the spermatozoa swimming on top of the partition wall video-recorded. A temperature difference of 2°C between the wells was maintained to stimulate thermotaxis. Alternatively, as a no-gradient control, both wells were kept at the same temperature, either 37°C or 39°C.

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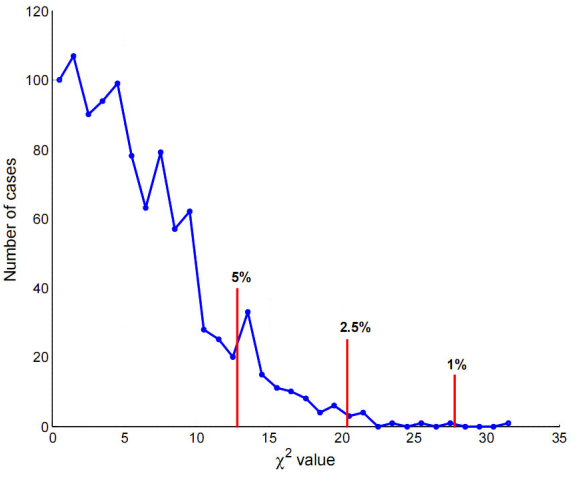
Appendix

χ^2 standardization

The critical χ^2 value with n degrees of freedom (df) at significance level α is the value that cuts off α% of the distribution from the upper tail of a χ^2 distribution with n df. Because the critical values found in the tables were very low relative to the magnitudes of our test statistics (as a result of huge sample sizes), we developed a method to obtain a critical value for a χ^2 statistic with 1 df, which takes the sample size into account. By definition, a critical value for a statistical test is obtained as a percentile of its null distribution. The null hypothesis will be represented by two control replicates within the same experiment, which should provide similar distributions of γ_{inst} values.

The first step was to take 30 pairs of control replicates, each having 10,000-50,000 γ_{inst} values from 28 experiments carried out under the same conditions (called the original samples). The next step was to use the computer-intensive bootstrap method (Efron and Tibshirani, 1993). The idea of bootstrapping is to create a large number of sub-samples from a given original sample by re-sampling with replacement (meaning that one value could be withdrawn several times). The re-sampling procedure can be repeated as many times as wished (Efron and Tibshirani, 1993), thus providing the user with many possible values of the test statistic, which can then be used in order to get the required percentile (cut-off point). For each original pair of samples of sizes *N1* and *N2,* we resampled (with replacement) 1000 pairs of samples of size N_{sub} where N_{sub} ranges from 5000 to *N1 or N2,* whichever is the smaller sample size, in jumps of 5000 and calculated the χ^2 statistic. So for each N_{sub} we now have 1000 values of the χ^2 statistic, which can be ordered, and percentiles can be found (1%, 5%, 10%, etc.). Fig. 4 is an example. We repeated the above procedure 30 times (that is, performed the steps for each original pair of samples separately), and thus obtained, for each value of N_{sub} 30 cut-off points for 5%, 30 cut-off points for 1%, etc. To summarize, after repeating all the steps for all the experiments, for each pre-set N_{sub} we had (approximately) 30 cut-off χ^2 values for each significance level. These 30 values were not normally distributed (Fig. 5), for which reason we used the median as a statistical characteristic of a cut-off χ^2 specific for a given significance level and a given sample size. The median cut-off χ^2 values were linearly related to the sub-sample size N_{sub} (Fig. 6), allowing us to use linear fitting functions to build a table of new empirical critical χ^2 values (Table 3).



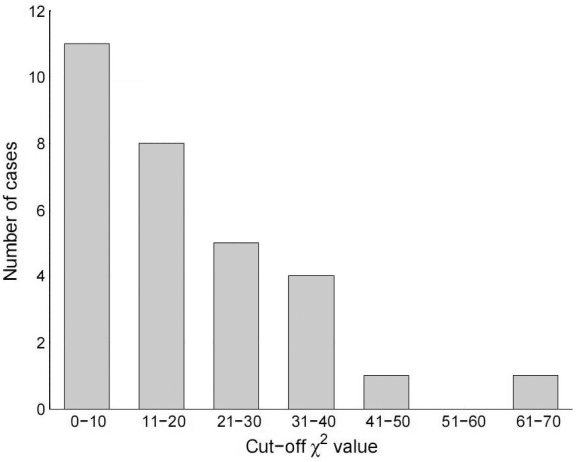


Fig. 4 (Left). Example of frequency distribution of χ^2 values. *The distribution was built for 1000 χ^2 values, which were computed for two subsamples of 10,000 points, drawn from two control replicates of a single experiment. Red lines indicate the cut-off points at 5%, 2.5% and 1% of the data from the upper tail of the distribution.*

Fig. 5 (Right). Frequency distribution of 30 cut-off χ^2 values. *The frequency distribution was calculated for 30 values, which determined the cut-off χ^2 at the 5% significance level from the distribution as in Fig. 4, specific for N_{sub} = 10,000.*

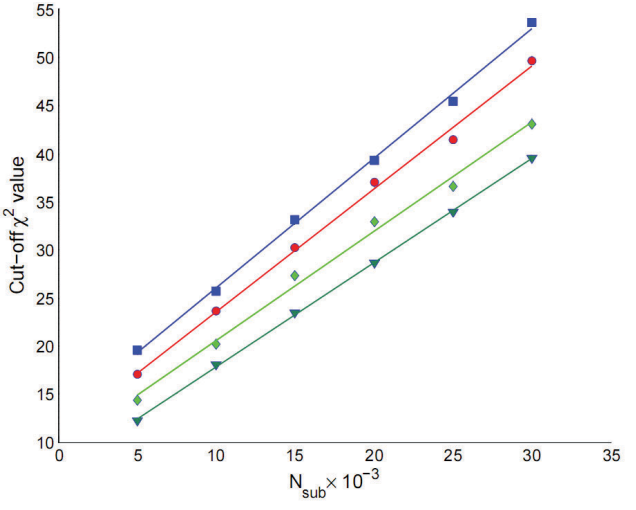


Fig. 6. Median values at different significance levels α plotted against subsample size and fitted with linear functions. *Significance levels and correlation coefficients are: blue, α = 0.001%, R = 0.993; red, α = 0.01%, R = 0.998; light green, α = 0.025%, R = 0.997; dark green, α = 0.05%, R = 1.00.*

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