

Flagellar and Ciliary Beating in Trypanosome Motility

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The single flagellum of *Leishmania* and *Trypanosoma* parasites is becoming an increasingly attractive model for the analysis of flagellar function—driven largely by the abundance of genomic and proteomic information available for the organelle, the genetic manipulability of the organisms and the importance of motility for the parasite lifecycle. However, as yet, there is a paucity of published data on the beating of any genetically malleable trypanosomatid species. Here we undertook an in-depth analysis using high-speed videomicroscopy of the beating of free-swimming *Leishmania major* cells in comparison to *Crithidia* species (for which there is some existing literature). In so doing, we describe a simple and generally-applicable technique to facilitate the quantitative analysis of free-swimming cells. Our analysis thoroughly defines the parameters of the expected tip-to-base symmetrical flagellar beat in these species. It also describes beat initiation from points other than the flagellum tip and a completely different, base-to-tip highly-asymmetric beat that represents a ciliary beat of trypanosomatid flagella. Moreover, detailed analysis of parameter interrelationships revealed an unexpected dependency of wavelength on oscillator length that may be the result of reversible constraint of doublet sliding at the tip or resonance of the flagellar beat. *Cell Motil. Cytoskeleton* 64:629–643, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

Eukaryotic cilia and flagella are mechanochemical oscillators that generate motile forces. They emerged early in eukaryotic evolution and are found in diverse organisms from microbes to metazoa (in which they fulfil several essential roles). In trypanosomes—including the medically important parasites *Trypanosoma brucei*, *T. cruzi* and *Leishmania* spp.—the single flagellum plays, in addition to motility, key roles in attachment to insect host epithelia [Tetley and Vickerman, 1985]; participation in mitochondrial DNA segregation [Robinson and Gull, 1991]; and cell division [Moreira-Leite et al., 2001]. Moreover, for *T. brucei*, flagellar motility has recently been demonstrated to be essential for survival of the parasite in the mammalian bloodstream form [Broadhead et al., 2006].

The recent publication of a complete genome sequence for the ‘TriTryps’: *T. brucei*, *T. cruzi* and *L. major* [Berriman et al., 2005; El-Sayed et al., 2005;

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Ivens et al., 2005], along with the availability of both reverse and (some) forward genetic tools, means that these organisms are appealing model organisms for analysis of many aspects of flagellar function and movement. This has been considerably enhanced by the publication of a trypanosome flagellar proteome [Broadhead et al., 2006]. However, at present, although some aspects of cell motility have been examined—for example, in recent qualitative report of the flagellar beat in *T. brucei* [Branche et al., 2006]—there exists no in-depth description of beating in these organisms against which hypotheses could be tested.

Non-parasitic species of the genus *Crithidia* are the only trypanosomatids to have received detailed attention in relation to flagellar beating. Specifically, *C. oncopelti* was used in several elegant early studies using cinematography [Holwill, 1965; Goldstein et al., 1970; Holwill and McGregor, 1974; Holwill and McGregor, 1976; Johnston et al., 1979]. These studies described the tip-to-base flagellar waveform seen in these organisms and defined several fundamental parameters for beat such as curvature, wavelength and beat frequency. However, it is unclear how far these data can be used to as models for the parasitic species for which there is now genomic and mutant information.

Here, we present an in-depth analysis of the flagellar beating of *Leishmania major*. This organism was chosen over the other two members of the TriTryp family as the nature of its beat is more amenable to quantitative study. We have compared this to two crithidial species—one (*Crithidia fasciculata*) containing a canonical trypanosomatid paraflagellar rod (PFR) and one (*C. deanei*) with a highly reduced PFR [Gadelha et al., 2005]—to test the general applicability of the results to other trypanosomatids and as a link to earlier work on *Crithidia*.

Two main techniques are used in studies of flagellar/ciliary beating: stroboscopic illumination and high-speed photography. While the former is convenient for viewing motion that is repeated at a uniform rate, the latter has a greater capacity for the observation of irregular or transient events. Here, we have used high-speed (fast-capture) videomicroscopy, capturing at 192 frame s^{-1} (sufficient to describe beating behaviour up to 96 Hz). This allowed us to not only describe the very rapid beating of *C. fasciculata* flagella (up to 70 Hz) but also a transient base-to-tip asymmetric ciliary beat that occurs spontaneously in all three species. We also describe a simple technique to facilitate the quantitative analysis of beats in free-swimming cells that is generally applicable to other motile cells. Using this technique, we have measured several fundamental parameters of the flagellar wave on a cell-by-cell basis. Analysis of the interdependencies of these parameters revealed an unexpected dependency on oscillator length that can be explained if

there is a degree of resonance to the flagellar beats in these organisms.

MATERIALS AND METHODS

Cells

Promastigotes of *Leishmania major* (Friedlin strain V) were cultured at 28°C in Medium 199 with Earle's Salts and L-glutamine (Gibco) supplemented with 40 mM HEPES, 10% (v/v) foetal bovine serum (Gibco) and 5 $\mu g ml^{-1}$ haemin. *Crithidia deanei* and *C. fasciculata* choanomastigotes were grown at 28°C in Brain and Heart Infusion medium supplemented with 5% (v/v) foetal calf serum.

Fast-Capture Videomicroscopy

All measurements were made on cells in growth medium (viscosity, 1.2×10^{-3} Pa s). Cell lines were grown to a density of 5×10^6 cells ml^{-1} and equilibrated to room temperature (22°C) for 1 h before the start of any experiment. Cells were then placed (in growth medium) onto slides and covered by a glass coverslip raised above the slide surface by tape of 50 μm thickness. This distance (~ 2 cell lengths) was chosen to be sufficiently large to allow cells to move freely without contacting the surfaces, but sufficiently small to approximate the cell motion to two dimensions by preventing large amounts of displacement in the z -axis. To prevent cell adherence, both slide and coverslip were pre-coated with poly-L-glutamate (Sigma). Finally, the edges of the coverslip were sealed to avoid capillary flow of liquid.

Videos were captured on a Zeiss AxioPlan II microscope attached to a MegaSpeed camera (MegaSpeed Co., Minnedosa, Canada) using the manufacturers software. All videos used in this report were recorded at maximum camera resolution (640×480 pixels; 8-bit depth). Frame exposure times were limited to 750 μs to minimise image blurring due to flagellar movement. For analysis of waveform, videos were captured at the fastest frame rate available for this resolution (192 frames s^{-1}). In accordance with Nyquist sampling theory [see Unser, 2000] this allows waves of up to 96 Hz to be analysed without 'foldback' artefacts in the frequency domain. We recorded ~ 40 individual cells from each species at this frame rate encompassing ~ 400 frames (2 s) each. Phase-contrast illumination was used and magnification of 100 \times for *Crithidia* spp. or 40 \times for *L. major*. For analysis of the swimming behaviour of the population, we recorded five videos for each species at 10 \times magnification using dark-field illumination and a capture rate of 100 frames s^{-1} over the period of 10 s for each species (1000 frames).

Image Processing and Statistical Treatment of Measurements

Pre-analysis image processing was performed using the publicly-available virtualdub software (www.virtualdub.org) with a deinterlace filter (Smart Deinterlacer; neuron2.net). All other processing, measurements and mathematical transformations were done using in-house programs to instruct the publicly-available ImageJ software (National Institute of Health, USA; rsb.info.nih.gov). These ‘macros’ are available from the authors on request. Co-efficients for n^{th} -order polynomials used to model cell displacement (see Results) were determined by a linear least-squares method [see Fisher, 1970] with the order of polynomial giving the line of best-fit determined by the eye. Minima and maxima in the transformed flagella during beating were used to determine mean amplitude and wavelength for the beats of individual cells, and distance along the flagellum between these two points was used as a measurement of half arc-wavelength. Reslicing of videos was used to measure beat frequency. Curvature measurements were made from splines fitted to flagella. Shear angle of any point along the flagellum is defined as the angle of intersection between the tangent to the flagellum curve at that point and the tangent at the base of the flagellum. Estimates of shear angle were made from splines using the angle of the longest axis of the cell body as a surrogate for the angle of the base of the flagellum, which lies at the base of the flagellar pocket just inside the anterior end of the cell.

Correlation analyses used the Pearson’s correlation coefficient as a test statistic. The significance of the value of the test statistic for each correlation was assessed by Monte-Carlo permutation (randomisation) tests, since these tests require no particular assumptions concerning the population distributions. Two null hypotheses were tested: (1) that there is no overall significant correlation between the parameters (tested by randomly permuting values of both between and within species subsets) and (2) that the correlation is product only of the intra-species parameter means (tested by randomly permuting all values only within the subsets). All tests were performed using the statistical programming package ‘R’ (The R Project for Statistical Computing; www.r-project.org), 10,000 permutations in all cases and 2-tailed P -values were estimated from the number of permutations having as strong or stronger values (either positive or negative) for the test statistic. As expected, tests using the non-parametric Spearman’s rank-correlation coefficient as a test statistic resulted in very similar P -values (data not shown).

RESULTS

A Method for Analysis of Freely Swimming Cells

The motion of a free-swimming flagellate cell has two components: (1) the beating of the flagellum (or flagella) and, (2) the resultant translational displacement of the cell. Little quantitative analysis of flagellar beating can be performed unless translational cell displacement can be removed, since the flagellar waveform at any one time-point can not be directly superimposed over the waveform at another time (Fig. 1A). One approach to address this is to physically impede cell motion, for instance by attaching the cell body to a substrate. The downside of this method is that close association to a substrate may, in itself, alter flagellar beating; the motion of a flagellum of a physically impeded cell does not necessarily reflect flagellar beating in freely motile cells. Here, we have used an alternative approach which does not suffer from the potential artefacts of cell impediment. In this approach, the beating of freely motile cells was recorded, and then cell displacement was computationally subtracted—resulting in cells that are ‘swimming on the spot’ against a moving background.

To subtract cell displacement without also removing aspects of flagellar beating requires a model of the movement of the cell. In this analysis we have used as convenient reference points the proximal and distal ends of the flagellum. The positions of these two points were measured over a specific time period. The motion of each point with respect to time ($x(t)$, $y(t)$) was then fitted to two n^{th} order polynomials of the form:

$$x(t) = a_0 + a_1t + a_2t^2 + \dots + a_nt^n$$

$$y(t) = b_0 + b_1t + b_2t^2 + \dots + b_nt^n$$

where a_i and b_i are constants. This models the movement of the cell through space with respect to time (Fig. 1B). In practice it was rarely necessary to use more than first order polynomials to effectively model cell displacement over the time-periods analysed (although the model is plainly scalable to more complicated translational motion). Images were then transformed to remove cell translation and rotated such that the principal axis of wave propagation lay along the x -axis (in the proximal-distal direction; Fig. 1C). Note that this technique does not require any part of the cell to be stationary with respect to flagellar beating, since no point becomes fixed in the process—only cell displacement is removed, not any oscillations of the cell/flagellum due to flagellar beating. Also, although we have modelled the movement of two reference points in this analysis (to define precisely the axis of wave propagation), only one point is necessary to model the direction of cell displacement at

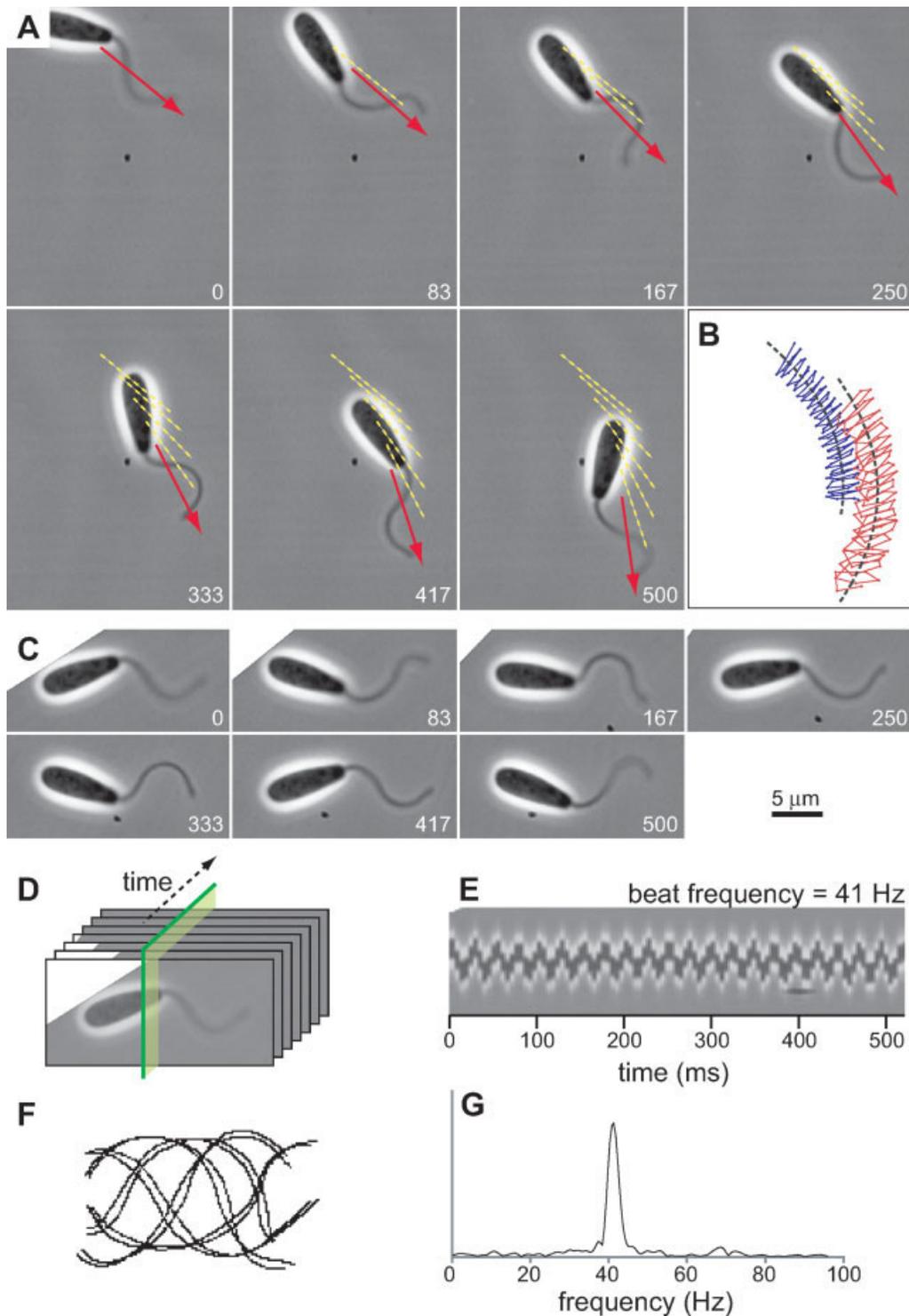


Fig. 1. A computational approach to separate cell displacement and flagellar beating of freely motile cells. (A) A montage of stills from fast-capture videomicroscopy showing untransformed cells. On each still, cell position and direction of motion is illustrated (red arrows) as well as those from previous stills (yellow lines). Time intervals (ms) are shown bottom right. (B) Plots of the movement of the proximal (blue circles) and distal ends (red diamonds) of the flagellum over 500 ms at 5.2 ms intervals. The centre points of both ends $\{x(t), y(t)\}$ mod-

elled as described in the text are also shown (dashed line). (C) Transforming the images in A to remove cell displacement results in cells that are 'swimming on the spot' enabling quantitative measurement of waveform properties. For example, reslicing the data at a fixed position along the x -axis (D) reveals the movement of that position with respect to time (E). (F) Superimposition of flagellum contours from the first 10 frames of Supplementary movie 2. (G) Shows a discrete Fourier transform of the flagellar beat in E.

any given time (atan $dy(t)/dx(t)$). The technique also provides an accurate quantitation of cell displacement speed.

Figure 1 and supplementary movie 2 illustrate the application of this technique to a free-swimming *Crithidia deanei* cell. The flagellar positions from the cell when 'swimming on the spot' can be directly superimposed (Fig. 1F) and alignment to the principal axis of wave propagation allows easy extraction of quantitative data (such as amplitude and wavelength) for the beat. Reslicing the video along the z -axis at any point along the flagellum (Fig. 1D) reveals the movement of this position with respect to time (Fig. 1E)—showing the regularity of the flagellar beat. This can be seen more clearly by transforming these data into the frequency domain by Fourier transformation (Fig. 1G) which also provides a convenient method for measurement of the beat frequency.

Comparison of Flagellar Beating in Three Trypanosome Species

Here, we present an analysis of flagellar beating in three trypanosomatid species: *Crithidia deanei*, *C. fasciculata* and *Leishmania major*. These species were selected because: (1) all three possess free flagella (not attached to the cell body for any great length) allowing easy observation; (2) the flagellar beating of each is planar, so the entire wave can be captured in a single focal plane and analysed as a 2-dimensional wave; (3) *Crithidia* spp. were the subject of earlier publications on flagellar beating and have the best studied motility of all the Trypanosomatidae [Holwill, 1965; Johnston et al., 1979] and (4) the genome of *L. major* has recently been sequenced to completion [Ivens et al., 2005]. This last point is important since, combined with the many reverse genetic tools available for this organism, it means that hypotheses about the influence of specific genes on flagellar beating can be readily tested in *Leishmania*. In all our analyses, we chose fast-capture over stroboscopic microscopy to allow the analysis of more complex and/or irregular beat behaviour.

Figure 2 illustrates the flagellar beating of the three trypanosomatid species (see also supplementary movies 2–4). In spite of a rather confusing taxonomy, *C. fasciculata* and *L. major* are the most closely related of the three species. Both are from the *Leishmania* clade [Gadelha et al., 2005] and possess a canonical paraflagellar rod (PFR) that runs nearly the full length of the axoneme. In contrast, *C. deanei* is from a separate clade of endosymbiont-containing trypanosomes in which the PFR has been drastically reduced and is present for only the proximal $\sim 1/3$ of the flagellum [Gadelha et al., 2005].

It is immediately apparent from the figure (and supplementary videos) that all three species execute a planar flagellar beat running tip to base, as previously

shown for *C. oncopelti*. This beating is symmetrical: both halves of the wave having equal and opposite curvature (data not shown). Moreover, for these free-swimming cells for which there is no impediment to cell motion, the curvature, wavelength and amplitude of flagellar waves did not detectably change as the wave travelled from tip to base (for example see Fig. 1F). This is different for the situation reported for *Trypanosoma brucei* [Branche et al., 2006] in which amplitude was reduced towards the proximal end, although this situation could be the result of cell attachment as much as dampening by the cell body. Obviously, all points along the flagellum beat with the same frequency, but with a difference in phase. The suggestion of a diminution of wave frequency toward the proximal end of the flagellum in *T. brucei* [Branche et al., 2006] seems rather improbable as it implies that adjacent points on the flagellum cycle in and out of phase with each other (i.e. it requires all points to be disconnected, which is clearly not the case).

We undertook a thorough quantitative comparison of the main features of the symmetrical flagellar beating observed in these species, the results of which are summarized in Table I. These data reiterate the similarities between the waveforms of the species. The major differences are in the beat frequencies—with *C. fasciculata* beating more than twice as fast as *L. major* (for example see Fig. 2). This is a reflection of a much greater propagation speed for the flagellar wave in *Crithidia* (rather than a reduced wavelength). Wavelengths and amplitudes are remarkably similar between the species, although the flagellum of *L. major* beats with an amplitude slightly greater than that observed for the *Crithidia* species.

Interestingly, there is little difference between the beat curvatures (or wavelengths) of the two *Crithidia* species. This is in spite of the presence in *C. fasciculata* of a substantial, paracrystalline PFR that has a cross-sectional area approximately equivalent to the axoneme itself [Gadelha et al., 2006]. Moreover, we find no evidence for a change in the curvature (or wavelength) of the *C. deanei* beat over the last $1/3$ of the flagellum which possesses a residual PFR (data not shown).

Trypanosomatid Flagella Beat With a 'Resonant' Wavelength

The relationships between the basic components of a wave contain information about the way the wave is formed. Since our analysis was performed on a cell-by-cell basis, it enabled us to look for such correlations in trypanosomatid flagella (see Fig. 3). There is a strong relationship between the translational movement of the cells and their beat frequency (Fig. 3A), but this correlation is the product of the positions of the species-specific means for these parameters and is not significantly contributed to by intraspecies trends (compare $p1$ and $p2$ in

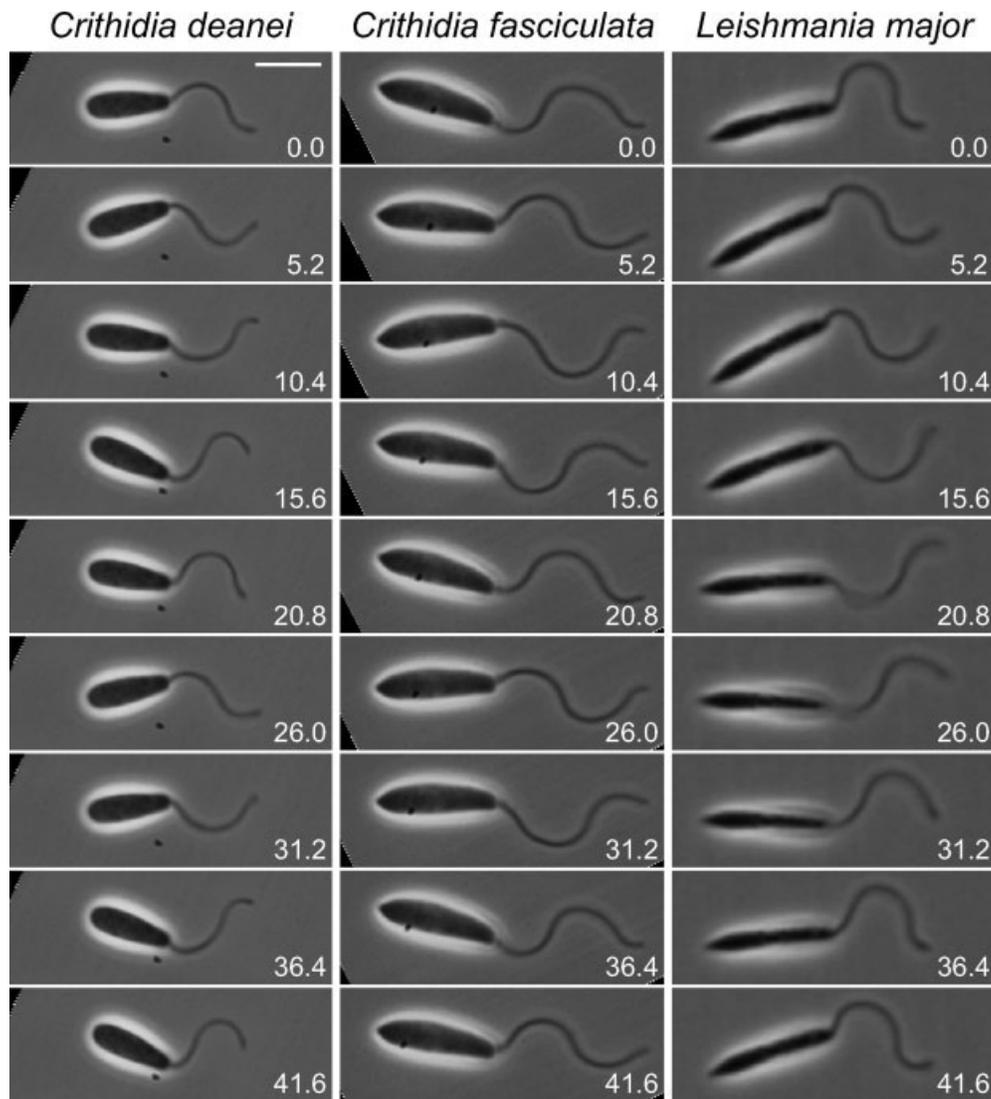


Fig. 2. Illustrations of the flagellar waveforms of *Crithidia deanei* (beat frequency = 41 Hz), *C. fasciculata* (58 Hz) and *Leishmania major* (27 Hz). Images show stills from fast-capture videomicroscopy that have been transformed to remove cell displacement (see Fig. 1). The *Crithidia* species complete ~ 2 waves over the times shown, whereas *L. major* completes only 1. Time intervals (ms) are shown bottom right. Bar, 5 μm .

Fig. 3A). When considering instead the speed of propagation of the wave (Fig. 3B), not only does the correlation to cell speed increase, but it is the result of both inter- and intra-specific correlation (p_2 in Fig. 3B). This makes good biological sense, since it is this motion, rather than the beat frequency itself, that provides the force to move the cell. The length of the flagellum does not influence beat frequency although there are clear differences in average frequency of beat for the species (Fig. 3C).

More interesting was the dissection of beat propagation. The beating of eukaryotic flagella is the product of the alternating activation/deactivation of dyneins.

Hence, one might expect that the properties of the beat would be defined by the characteristics of any individual part of the flagellum through which the wave propagates (stiffness, speed of dynein activation/recovery, cooperative activity of adjacent dyneins, etc.)—i.e. that wave propagation speed would be one of the fundamental features of the flagellum for any given species. This requires there to exist a strong negative correlation between wavelength and beat frequency (since wave propagation speed is a product of wavelength and beat frequency). Such a relationship has been observed for sea urchin, cricket and bull sperm flagella [Rikmenspoel, 1978]. However, for our dataset, no such correlation exists,

TABLE I. Mean Parameters (\pm s.e.m.) for Flagellar Beating

	<i>C. deanei</i>	<i>C. fasciculata</i>	<i>L. major</i>
Cell parameters			
Cell length (μm)	7.4 ± 0.2	11.1 ± 0.3	12.5 ± 0.3
Flagellum length (μm)	13.1 ± 0.4	15.1 ± 0.5	16.4 ± 0.6
Cell propagation speed ($\mu\text{m s}^{-1}$)	45.6 ± 1.5	54.3 ± 2.6	36.4 ± 2.0
Beat parameters			
Beat frequency (Hz)	40.5 ± 0.8	60.0 ± 2.3	24.5 ± 0.8
Amplitude (μm)	2.2 ± 0.05	2.2 ± 0.07	2.9 ± 0.07
Wavelength (μm)	11.7 ± 0.2	11.6 ± 0.2	11.9 ± 0.3
Arc-wavelength (μm)	17.0 ± 0.4	16.0 ± 0.4	18.0 ± 0.6
Wave propagation speed ($\mu\text{m s}^{-1}$)	466 ± 12	680 ± 28	291 ± 4
Curvature ($\text{rad } \mu\text{m}^{-1}$)	0.28 ± 0.006	0.25 ± 0.006	0.30 ± 0.009

either between or within species (Fig. 3D). Surprisingly, instead we found a strong positive correlation between wavelength and flagellum length (Fig. 3E), both for each species separately and for the dataset as a whole. We considered that this might be caused by an adjustment in wavelength such that there is one complete beat per flagellum length—i.e. that these flagella beat at a fundamental or resonant wavelength. Figure 3F shows that there is indeed a strong linear relationship between the arc-wavelength (the distance from one wave to the next along the path of the flagellum) and the flagellum length for any individual cell, with one beat occurring on each flagellum. On longer flagella, lengthening of the wave is accompanied by both an increase in wave amplitude (Fig. 3G), and a decrease in curvature (Fig. 3H) resulting in roughly the same overall change in angle for each half-wave, regardless of wavelength (Fig. 3I). Thus, for the species studied here, the flagella exhibit wavelengths defined not by cross-sectional characteristics of any one point, but by the length of the oscillator as a whole.

The occurrence of an integer number of beats per flagellum means that the tip and base of the flagellum are roughly in phase, and there is thus a reduced shear at the tip during flagellar beating compared to other points along the length of the flagellum (Fig. 4A). However, despite the strong correlation between flagellum length and arc-wavelength described above, temporal variation in the effective wavelength means that there is not always zero shear at the tip, the shear angle of which can be up to ~ 0.8 rad in cells beating tip-to-base (Fig. 4A and data not shown). This can also be seen in Fig. 2 and the Supplementary movies (observe the difference in angle between the cell longitudinal axis and the tangent of the tip).

Beat Initiation at Points Other Than the Flagellum Tip

One interesting feature of the beating of trypanosomatid flagella is that they are able to initiate beating from the flagellar tip, rather than the proximal end as in

most other organisms. This has led to the proposal that there might be some tip initiating structure that provides resistance to doublet sliding in a manner similar to the basal body [Woolley et al., 2006]. However, while it is undoubtedly true that the flagellar beat initiates near the tip, a careful observation of the cells reveals that the most distal 1–2 μm often appear not to bend (see Supplementary movies). Moreover, we observe that, in a minority of free-swimming cells, beat initiation is clearly separate from the flagellum tip (see Supplementary movie 5). These cells are able to switch between (near-to) tip initiation and a separate beat initiation site several microns from the tip (Supplementary movie 5). However, in all cases beat termination is still at the flagellum proximal end.

Cell propagation speeds are generally lower when waves are initiated at points other than the ends ($19 \pm 4 \mu\text{m s}^{-1}$ for 10 *L. major* cells), which is unsurprising given that less of the flagellum is actively beating, although it may reflect the fact that beat frequencies are also reduced (14 ± 1 Hz). Significantly, when beats initiate at points other than the tip, the wavelength is reduced such that the arc-wavelength again equals the effective length of the oscillator (i.e. the length of actively beating flagellum), as shown in Figure 5. This provides strong support for the above proposal of a resonant wavelength for tip-to-base beating in these cells. Since the non-beating part of the flagellum is not greatly curved, these resonant waves also tend to reduce the shear at the flagellum tip, but as in the case for beating initiating from the tip, some shear is still observed (data not shown).

Ciliary Beating of Trypanosomatid Flagella

During our observation of the motion of these species we noticed another, quite distinct type of beat to those described above. This beat is also planar, but is propagated in the opposite direction (base to tip) at a much lower frequency. It is also highly asymmetrical—consisting of two half waves with dissimilar curvatures

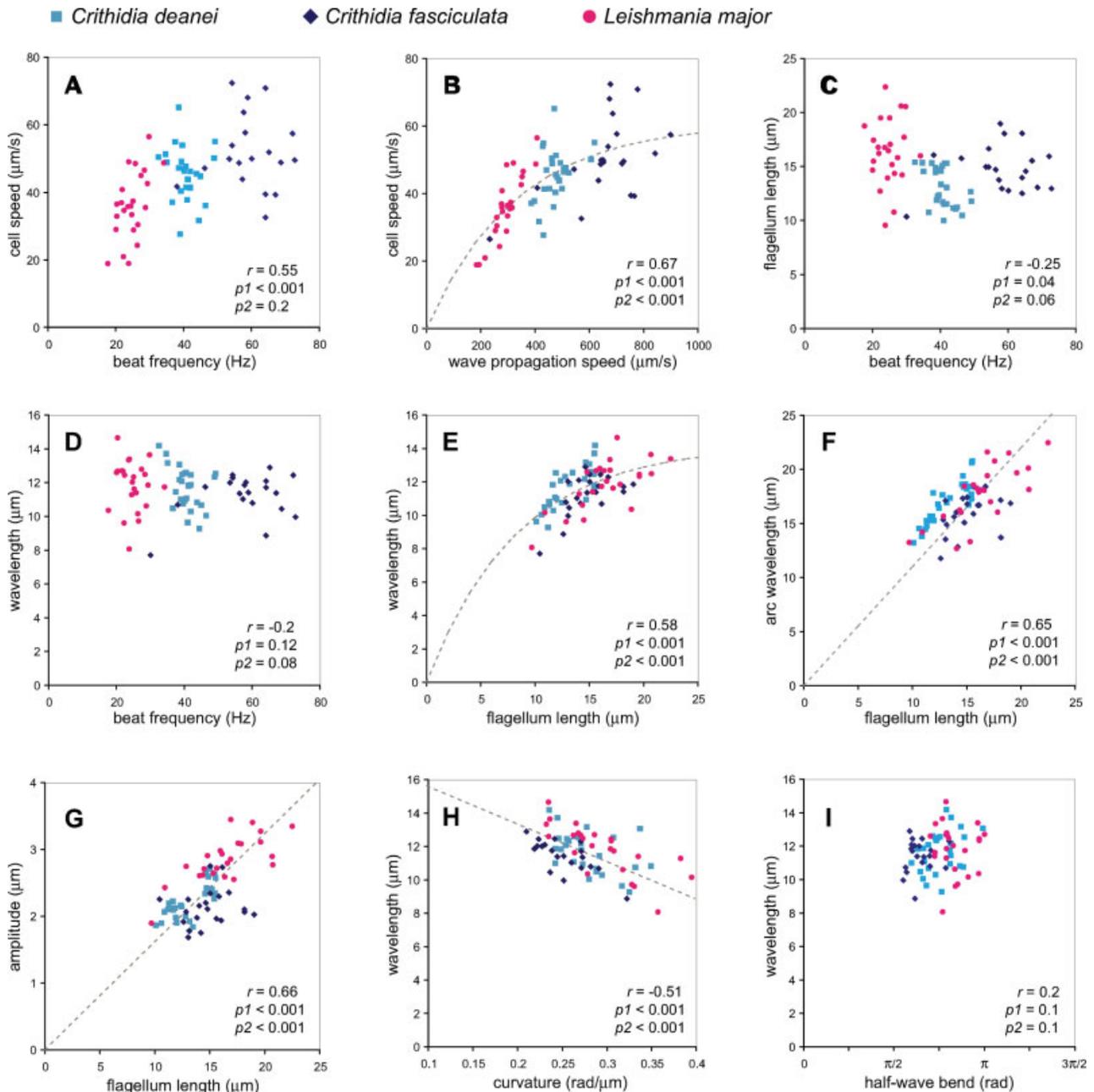


Fig. 3. Relationships between some of the components of the flagellar beats of *Crithidia deanei*, *C. fasciculata* and *Leishmania major*. The significance of the test statistic, the Pearson's correlation coefficient (r), was assessed by Monte-Carlo permutation tests, either permuting all values for a given parameter ($p1$; testing the strength of the overall correlation) or permuting values only within the species

subsets ($p2$; testing the strength of the correlation regardless of the subset means). P -values ≤ 0.003 are considered significant (2-tailed test with Bonferroni correction for multiple tests). Dotted lines show lines of best fit [$y = ax$, $y = ax + b$ or $y = a(1 - e^{-bx})$] where a significant correlation was observed. $N = 27$ (*C. deanei*); 21 (*C. fasciculata*); 24 (*L. major*).

and wavelengths: an apparent ciliary beat (see Figs. 4B, 6, and 7 and Supplementary movies 6–8).

The trypanosomatid ciliary beat initiates apparently spontaneously in all three species, and can be divided into four phases: (1) a cell performing planar

symmetrical tip-to-base (flagellar) beating interrupts initiation of the tip-to-base beat, and the last flagellar wave runs to the base; (2) there is then a pause during which no movement is observed; (3) ciliary waves are initiated at the base moving all the way along the flag-

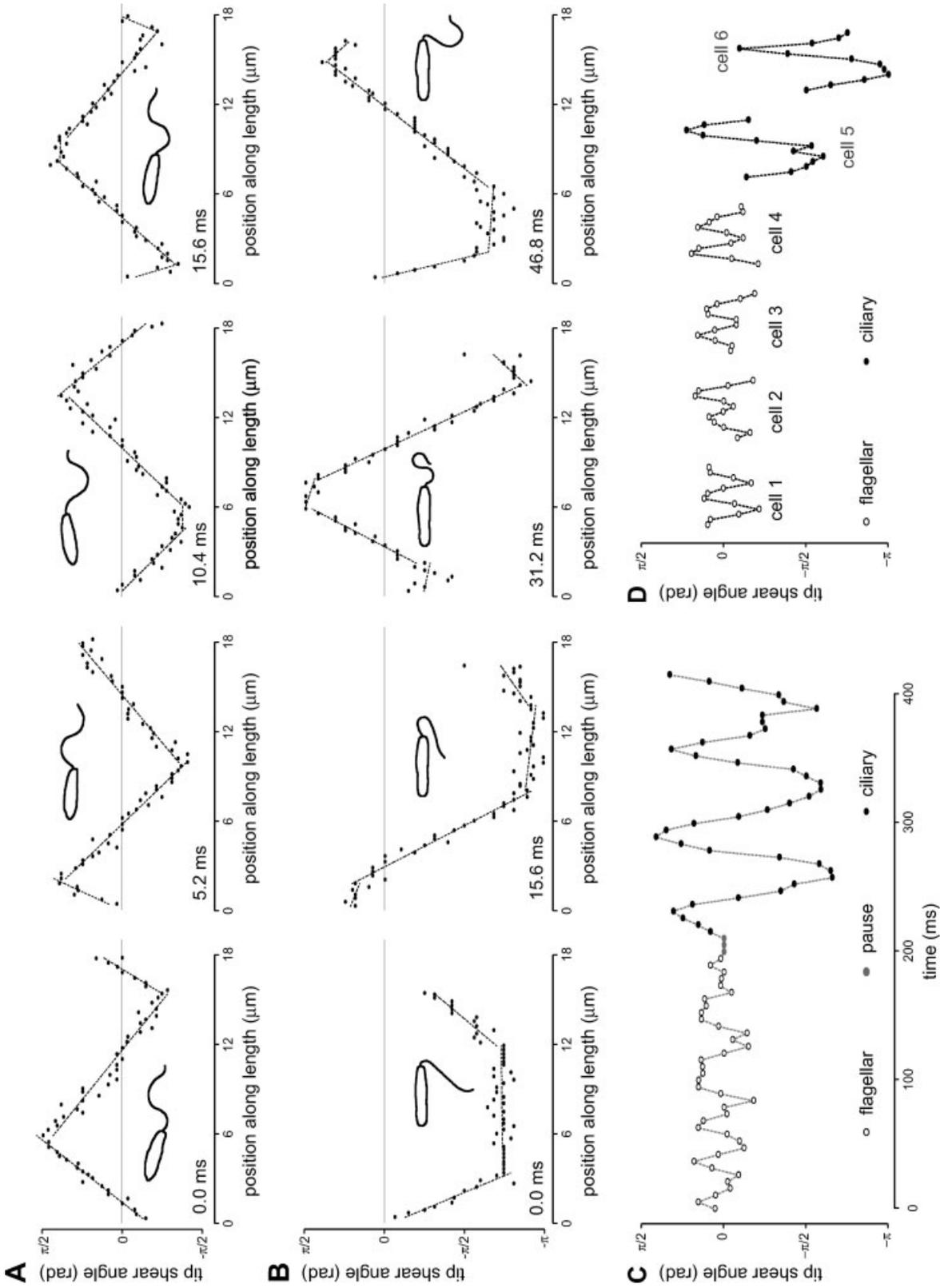


Fig. 4. Shear angle as a function of the length along the flagellum for four frames covering ~ 1 complete flagellar beat cycle (A) or ~ 1 complete ciliary beat cycle (B) in *Crithidia fasciculata*. The shear angle was estimated from the tangent to the flagellum at each point relative to the cell body longitudinal axis (used as a surrogate for the axis of the basal body). Linear lines of best fit have been superimposed on the data (dotted lines). Plots are separated by the intervals shown at its bottom left. Each plot contains a graphical representation of its respective cell (drawing). (C) Shear angle as a function of time at the flagellum tip for a *C. deanei* cell during flagellar (open circles) and ciliary (filled circles) beating. Grey circles represent the short 'pause' corresponding to the transition between beat types. (D) Shear angle at the flagellum tip for six *C. deanei* cells performing either flagellar (cells 1-4, open circles) or ciliary beating (cells 5-6, filled circles). For each cell 12 timepoints are shown at a spacing of 5.2 ms.

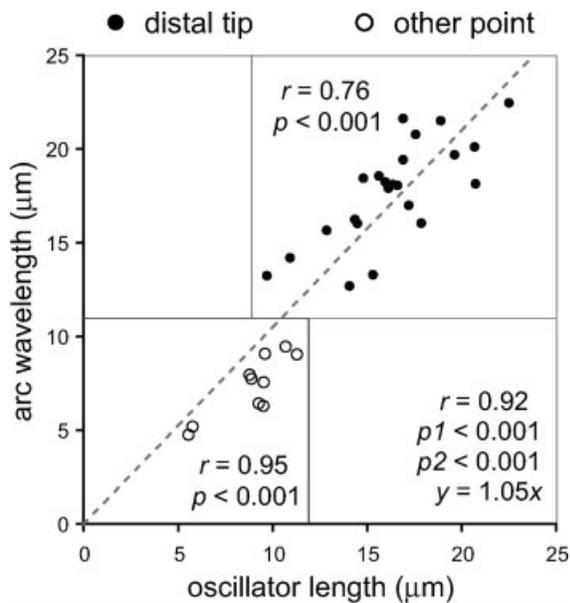


Fig. 5. Strong correlation between oscillator length (length of flagellum actively beating) and arc-wavelength in *Leishmania major* cells—one complete beat per oscillator. Symmetrical flagellar waves propagating from (near to) the distal tip (closed circles), or at other points (open circles) are shown. See legend to Fig. 3 for explanation of *P*-values.

ellum to the tip; (4) initiation from the tip is halted, the last ciliary wave reaches the tip and, finally, cells reinitiate the tip-to-base flagellar beat. There is variation in both the length of the pause (short and rare in the *Crithidia* species, up to 80 ms in *L. major*) and the number of ciliary beats performed (1 to ~ 15 for all species). However, the outcome is very consistent: the ciliary beat produces very little translational motion of the cell, but results in a change of cell orientation (the cell body rotates $\sim 25^\circ$ for each beat performed) such that the cell swims in a different direction when flagellar beating reinitiates.

The properties of the ciliary beat in the three species analysed are summarized in Table II. The beat frequency and speed of propagation of the ciliary beat are 25–35% that of the respective flagellar beats for the same species. In trypanosomatids, the ciliary beat initiates with the sharper-curved principal (P) wave, followed directly by the reverse (R) wave, which has a gentler curvature value. Both P and R waves are propagated at the same speed, i.e. the two do not catch up or lag behind one another. The ciliary wave as a whole, however, accelerates as it travels along the flagellum, although its highest value is still much lower than the flagellar wave propagation speed. In the two *Crithidia* species, the R wave consistently runs off the flagellum before initiation of the next beat whereas in *L. major* beat initiation occurs before the previous wave has left

the flagellum (Fig. 7, right panels). In contrast to the flagellar beating of these organisms, there is no apparent resonant wavelength to the ciliary beat in any of the three species and this highly asymmetric beat results in a large shear at the flagellum tip (up to ~ 3 rad; Figs. 4B–4D). In no cells did we observe both flagellar and ciliary beats concurrently.

DISCUSSION

Quantitative analysis of flagellar beat phenomenon often require the removal of translational cell displacement such that different time points in the cycle can be superimposed. Physical attachment of the cell body provides one method for doing this, although the restraint of natural cell movement combined with the proximity of the attachment substrate can influence beat. Post-capture alignment of the cell body provides another, but does not allow for oscillatory movement of the cell itself in response to beat (hence many features are no longer quantifiable). Here, we have used an alternative method for the analysis of freely swimming cells by computational removal of cell displacement without removing oscillatory motion of the beat. We have used the technique on trypanosomes, although it is clear to see how it could be applied to almost any organism/cell in which beating is reasonably consistent in terms of cycle duration.

We have used this technique to thoroughly describe the beating of three trypanosomatid species with free flagella (for ease of interpretation): *Crithidia deanei*, *C. fasciculata* and *Leishmania major*. For the latter, the recent publication of its complete genome sequence, the nature of its beat (see below) and the availability of both reverse and forward genetic tools, make it an appealing model organism for the analysis of flagellar beating.

One of the morphological features used in our choice of organisms was the presence/absence of a canonical (i.e. large and paracrystalline) paraflagellar rod structure. RNAi-mediated knockdown in the levels of one of the major PFR proteins in *Trypanosoma brucei* results in ablation of cell displacement, although not all movement of the flagellum is lost [Bastin et al., 1999]. Whereas the *L. mexicana* homologous knockout still swims, although with much reduced speed [Santrich et al., 1997]. It has been assumed that this (often very extensive) structure adds considerably to the stiffness of the axoneme. While in no way conclusive, the similarity between the beats of (PFR-containing) *C. fasciculata* and (largely PFR-less) *C. deanei* argues against this—as does the lack of observable differences between the curvature of the proximal and distal ends of *C. deanei* flagella. However, removal of the PFR from species that usually possess it is clearly detrimental to beat forma-

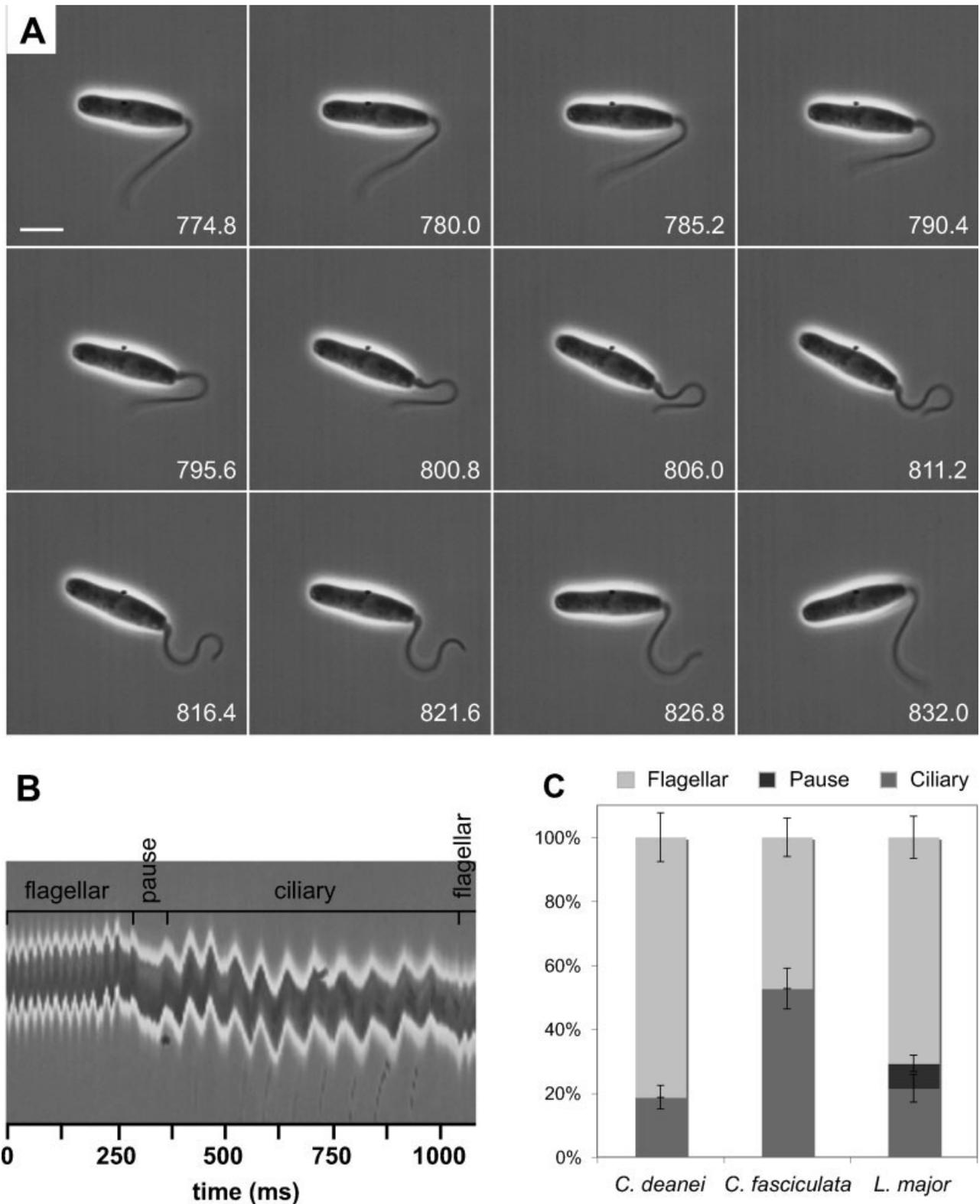


Fig. 6. Ciliary base-to-tip beating in trypanosomatid flagella. (A) A montage showing one complete ciliary beat taken from Supplementary movie 6. Time intervals (ms) are shown bottom right. Bar, 5 μ m. (B) All frames of the same video resliced (through the flagellum base) to show beat frequency. (C) The distribution of swimming behaviours for *C. deanei*, *C. fasciculata* and *L. major* populations. For each species \sim 100 cells were observed over 100 ms. Beat types are: flagellar (symmetrical tip-to-base), ciliary (asymmetrical base-to-tip) and 'paused' (the transition from flagellar to ciliary). Bars represent standard errors.

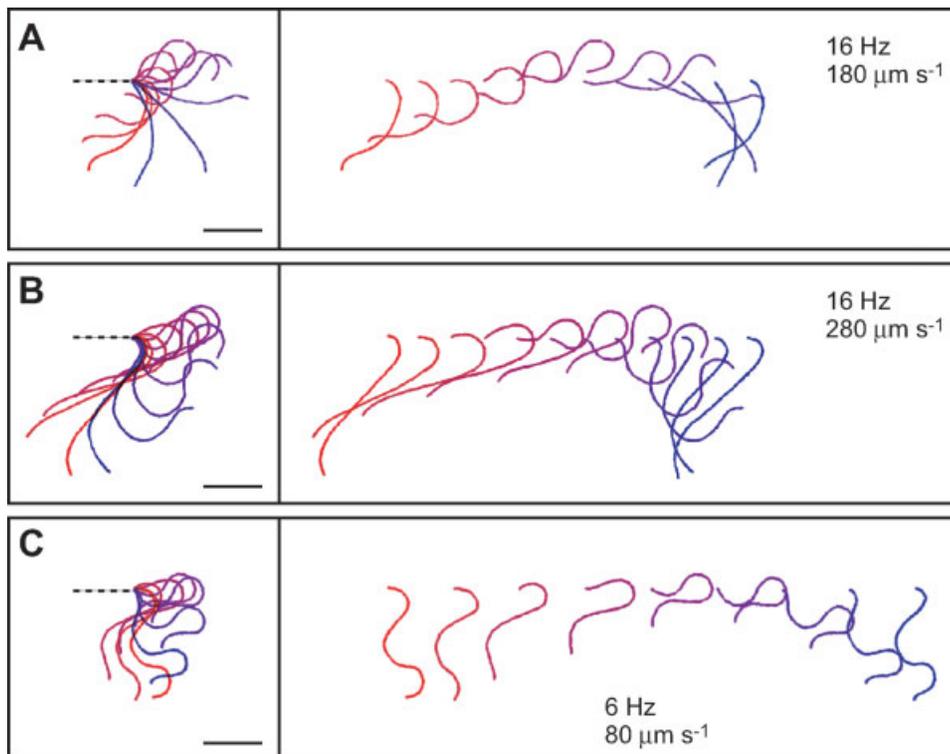


Fig. 7. Representations of one complete ciliary cycle for: (A) *Criethidia deanei*, (B) *C. fasciculata* and (C) *Leishmania major*. Beat lines are separated by 5.2 ms (*Criethidia* spp.) or 10.4 ms (*L. major*) and a colour gradient (red to blue) added to follow progress through the cycle. Each cell body has been fixed along the x -axis (dashed line). In the right panel time is also represented by a displacement along the y -axis. Note the difference in curvatures for the P wave (initiated first) and the R wave (immediately after). Bars, 5 μm .

TABLE II. Mean Parameters (\pm s.e.m.) for Ciliary Beating

	<i>C. deanei</i>	<i>C. fasciculata</i>	<i>L. major</i>
Cell parameters			
Change in direction (degree beat ⁻¹)	22 \pm 2	19 \pm 4	27 \pm 4
Beat parameters			
Beat frequency (Hz)	12 \pm 1	21 \pm 4	6 \pm 1
Wave propagation speed ($\mu\text{m s}^{-1}$)	126 \pm 11	230 \pm 45	70 \pm 4
P wave curvature (rad μm^{-1})	0.85 \pm 0.02	0.60 \pm 0.03	0.85 \pm 0.02
R wave curvature (rad μm^{-1})	0.60 \pm 0.07	0.36 \pm 0.02	0.60 \pm 0.02
Ciliary/flagellar parameter ratios			
Beat frequency	0.30	0.35	0.24
Wave propagation speed	0.27	0.34	0.24

tion. The suggestion that the PFR in *T. brucei* may contribute to the helical morphology of the cell is intriguing [Branche et al., 2006], although it does not explain the loss of motility in *Leishmania* PFR knockouts since these cells have a 2-dimensional (i.e. planar) beat. Moreover, the cell bodies of trypanosomatids with free flagella [for examples see Attias and de Souza, 1986; Sant'Anna et al., 2005] and also bloodstream-form *T. brucei* PFR knockdown cells [Broadhead et al., 2006] present a helically twisted shape. The finding of metabolic regulators in the PFR of *T. brucei* suggests that this structure may be a path for energy transduction rather than simply a physical stiffener [Pullen et al., 2004] and thus it may be the disruption to the metabolic environment of the flagellum that alters beat formation. Alterna-

tively, anisotropy in the stiffness of the PFR might regulate beat plane (as a functional analogue to the proposed role of the 5–6 bridge in sea urchin sperm, [Cibert, 2002]). In agreement with this, from our observations of live *T. brucei* cells it appears that the flagellar beat acts across the cell body—providing circumstantial evidence that the axis of bending is parallel to the plane defined by the microtubules of the central pair (since these are also roughly parallel to the cell surface).

Resonant Flagellar Wavelengths

One of the features identified here for flagellar beating of trypanosomatids is that wave propagation speed is relatively inconstant for each species and there is no reciprocal relationship between beat frequency and

wavelength. Of course, this could be a reflection of subtle differences between the characteristics of individual flagella. However, we find a very strong linear relationship between the length of the wave (arc-wavelength) and the flagellum length—indicating that there is a different process at work.

If one views the axoneme as a free ‘medium’ through which flagellar (or ciliary) waves may propagate, then the properties of this ‘medium’—axonemal stiffness, dynein activation/recovery times, co-operativity, environmental viscosity, etc.—would be expected to dictate the speed at which the waves propagate, analogously to sound waves propagating with a speed determined by the properties of the substance through which they move. This does not appear to be the case for the flagellar beating of *Crithidia* and *Leishmania* species. Instead, these flagella behave more akin to a tuning fork than sound travelling freely in a medium. Each flagellum beats with ~ 1 complete wave per flagellum (the fundamental wavelength) since this is a position of resonance for the oscillator as a whole. This observation of resonant wavelengths is considerably strengthened, in our view, by the observation of flagellar beat initiation at points other than the flagellum tip. In these situations, the new wavelength is considerably shorter than for tip-initiation such that, again, the arc-wavelength is equal to the oscillator length. Interestingly, the curvature and amplitude for any wavelength seems to adjust such that the two half waves complete approximately the same bend.

So what causes the flagella of these species to beat at resonant wavelengths? The observation of adjustment in arc-wavelength of flagella argues against the proposition that flagellum length is controlled in these species by beat wavelength—since for any individual flagellum, beat wavelength is variable and set by the length oscillating rather than the total flagellar length. A second possibility is that resonant wavelengths are the result of constraint of doublet sliding at the flagellum tip. Such constraint would effectively enforce only an integer number of beats per flagellum, since only if both ends of the axoneme are in phase is there no resultant shear at the flagellar tip (assuming there is no microtubule sliding at the basal body and no twisting of the axoneme). Woolley et al. [2006] have recently described a structure at the tip of *Crithidia* flagella which could provide such a constraint of tip sliding—although it is not yet known if it fulfils such a role or is associated with some other flagellar function (such as intra-flagellar transport, or flagellum building/maintenance). Interestingly, since each flagellum does not continuously describe a perfect single wave, we observed that there can still be a difference in the tangent to the proximal and distal ends of a flagellum of up to ~ 0.8 rad. This could be explained by cryptic beating within the flagellar pocket, although the amount

necessary to completely remove shear from the tip seems unreasonable, and the expected amount of tip ‘tilting’ that would result from this magnitude of shear (~ 120 nm) has been observed in electron micrographs [Woolley et al., 2006]. Thus if the tip structure is resistive to doublet sliding, it is only partially so. A third possibility is that there is a degree of internal reflection or feedback in the flagellar beat that sets up resonance. In this context it is notable that the flagella of *Crithidia* and *Leishmania* are able to propagate both tip-to-base and base-to-tip beats.

In comparison to other parameters of flagellar beating, the number of waves per beat has not been an extensive subject of study. In bull sperm and *Chlamydomonas*, the beat appears to have, on average, one wave per flagellum [Rikmenspoel, 1962; Janh and Bovee, 1967]—although a correlation between individual flagella and the number of arc-wavelengths has not, to our knowledge, been investigated for these cells. Conversely, in mouse and sea urchin sperm, the number of waves is reported to be ~ 1.2 and ~ 1.5 respectively [Brokaw, 1965; Phillips, 1972; Cosson, 1996]. The situation in most organisms is slightly complicated by the fact that the tip of the flagellum does not always propagate waves, meaning that the effective oscillator length is not necessarily the full flagellum length (see below). However, even with such caveats, for sea urchin and mouse sperm at least it is unlikely that the flagellum beats with a resonant wavelength. Whether caused by resistive structures at the flagellar tip or a degree of beat resonance, it may be that resonant wavelengths are only a feature of flagella that can propagate waves in both tip-to-base and base-to-tip directions. Interestingly, in theoretical fluid dynamic models of flagellar beating, there is a predicted optimum in propulsive efficiency for flagella propagating between 1.0 and 1.5 wavelengths [Dresdner et al., 1980]. Hence, the relationship between wavelength and oscillator length observed in trypanosomes may function to ensure that these flagella are beating near optimally whatever their length.

Beat Initiation at Points Other Than the Flagellum Tip

When observing the free-swimming species reported here, we noticed that waves could initiate from points other than the flagellum tip. This is interesting for what it implies regarding the initiation event itself. It has been proposed that tip-to-base initiation in trypanosomatids might require a capping structure at the flagellar tip to provide resistance to doublet sliding in a manner analogous to the basal body. There is undoubtedly a structure at the tips of *Crithidia* flagella [Woolley et al., 2006], however, these structural data alone can not determine

whether it represents a resistive cap, or is associated with some other function. The data presented here for free flagella support the micromanipulation experiments of Holwill and McGregor [1974] showing that physically restrained or dissected *C. oncopelti* flagella are able to reinitiate flagellar beating from the restraint/cut point (albeit after a delay). The reinitiation of beat from an injured flagellum might be a consequence of new cap formation. However, the rapid switching of beat initiation from tip to more proximal points along the flagellum (and back) that we observe in free flagella are unlikely to be the result of a relocation of a large structure. Since the non-beating (distal) portion of these flagella is not very curved, the initiation of beats part-way along the flagellum with a resonant wavelength does not conflict with the idea of a tip structure that is resistive to doublet sliding (since the tip shear is still minimised), although it shows that it is not necessarily required for beat initiation.

The Trypanosomatid Ciliary Beat

Alongside the symmetrical flagellar beat, we find that all three of the species observed also make a spontaneous base-to-tip ciliary beat. This beat (as is characteristic of ciliary beating) is highly asymmetric, consisting of a tight P wave followed immediately by a less-curved R wave and both are propagated at a much slower speed than for flagellar beating. The existence of a strict ratio between the beat frequencies and wave propagation speeds of the ciliary beat and the respective flagellar beat for the same species is intriguing, but we do not know its significance (if any). What is clear is that, in contrast to the flagellar beat of these species, the ciliary beat does not have a form with a wavelength sufficient for one complete cycle per flagellum and does not reduce doublet sliding at the tip. In fact, the largest tip shear angles that we observed (~ 3 rad) during ciliary beating would require a displacement of one side of the tip relative to the other ('tilting') of ~ 450 nm.

The finding of base-to-tip beats in trypanosomatids is not unprecedented: in his analysis of *C. oncopelti*—an endosymbiont-containing species that is very closely related to *C. deanei*—Holwill described a low-frequency asymmetric wave reversal [Holwill, 1965]. We propose that the asymmetric wave reversal described by Holwill and base-to-tip beating described here for *C. deanei*, *C. fasciculata* and *L. major* are the same motion and represent a true ciliary beat. Interestingly, the wave reversal behaviour, although apparently spontaneous, is encouraged in *C. oncopelti* by placement of the cell into an increased viscosity environment [Sugrue et al., 1988], or by severing of the flagellum near the tip [Holwill and McGregor, 1974]. These data, combined with the fact that the ciliary beat results in little translational motion

but causes a change in swimming direction, make it appealing to speculate that this might constitute an obstacle-avoidance response—although that neither the earlier work of Holwill and co-workers nor the work presented here directly address this possibility. However, it is easy to see how a signalling cascade triggered when the flagellar tip encountered an obstruction might either transiently release a resistive tip structure or interfere with beat propagation causing a cessation of tip-to-base beating. Base-to-tip beating would then take over for a short time, altering the cell orientation, until tip initiation recovered and forward motion restarted.

It is worth mentioning that we have never seen during our analysis of the three species used here the base-to-tip symmetrical flagellar beat seen (admittedly infrequently) by Holwill in his analysis of wildtype *C. oncopelti* [Holwill, 1965]. This may be due to species-specific differences, but it is also plausible that the flagella of *C. deanei*, *C. fasciculata* and *L. major* [which can so easily switch between flagellar and ciliary beating] are also able to maintain other beat types under different conditions.

Recently, there has been an increase in interest regarding motility of the related trypanosome, *T. brucei* [Branche et al., 2006; Davidge et al., 2006; Ralston et al., 2006]—accentuated by the finding that flagellar motility is essential for the viability of the bloodstream form of the parasite [Broadhead et al., 2006]. *T. brucei* is also an attractive model for flagellar studies since the levels of gene expression in the organism can be manipulated by well-established RNAi techniques [Ngo et al., 1998], unlike *L. major*, in which the RNAi mechanism appears to be absent [Robinson and Beverley, 2003]. Unfortunately, studies of the flagellum beat—as opposed to cell displacement—in this organism are hampered by the attachment of the flagellum to the cell body and the helical nature of the cytoskeleton (reflected in a 3-dimensional flagellar beat). A bimodal 'tumbling'/swimming behaviour has been described for insect-form trypanosomes [Hutchings et al., 2002], and recently Branche et al. have reported cell re-orientation caused by wave reversal in the same cells [Branche et al., 2006]. Work from our laboratory suggests that base-to-tip propagation of waves in *T. brucei* is also spontaneous and asymmetric (N. Portman and H. Farr, personal communication) and we propose that it represents the same ciliary beat as described here for *C. deanei*, *C. fasciculata* and *L. major*. We believe that the data presented here will provide a good descriptive background against which hypothesis about flagellar beating in trypanosomes might be tested.

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