Quantitative observations of flagellar motility of capacitating human spermatozoa

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For technical reasons, sperm head movement is assessed in kinematic analysis, while flagellar movement is the determining factor of head movement, not vice versa. It follows then that the development of new kinematic values to describe the movement of capacitating human spermatozoa should include the analysis of their flagellar movement. The aim of this study was to establish quantitative differences between flagellar movement patterns of hyperactivated and non-hyperactivated spermatozoa which could then be used in the evaluation of new centroid-based kinematic values. Spermatozoa were prepared by swim-up from semen into culture medium supplemented with 30 mg/ml human serum albumin. Sperm movement was recorded in 50 µm-deep chambers using a 200 Hz video system. Sperm movement was classified based on flagellar movement, with 24 non-hyperactivated and 26 hyperactivated spermatozoa included in the study. Flagellar analysis was performed using both a semi-automated analysis system (SIAM FLAG; 30 images at 200 Hz) and manual methods (100 Hz). Hyperactivated spermatozoa had significantly larger flagellar beat angles (≥87°) and significantly lower flagellar beat frequencies (≤29.4 Hz) than non-hyperactivated human spermatozoa. In addition, the flagellar wave amplitude was significantly greater and the bend diameter significantly smaller for hyperactivated spermatozoa in the proximal region of the flagellum (up to 20 µm from the head–midpiece junction). The velocity of the hyperactivated wave was low in this region, although it was significantly slower than the non-hyperactivated wave in all regions of the sperm tail.

Key words: flagellar/human/hyperactivation/kinematics/spermatozoa

Introduction

The analysis of sperm movement is an integral part of sperm function testing in infertility diagnosis, as well as in reproductive toxicology investigations and in quality control procedures (Schrader et al., 1991; ESHRE Andrology Special Interest Group, 1996). Sperm kinematic analyses are carried out routinely using computer-aided sperm analysis (CASA) instruments which monitor the movement of the sperm head and then analyse different aspects of its reconstructed trajectory. These analyses are typically performed at image sampling frequencies of 25–60 Hz, depending on the video system used (Mortimer, 1994).

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The aspects of sperm movement analysed by CASA include the velocity of motion of the sperm head, such as curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP), as well as the amplitude of lateral head displacement about the direction of travel (ALHmean and/or ALHmax) (Boyers et al., 1989). These kinematic parameters were derived from the study of sperm movement in seminal plasma and resulted from detailed analyses of both head and flagellar movement (David et al., 1981; Serres et al., 1984). The centroid-based values were found to relate closely to the flagellar movement patterns. So while centroid-derived kinematic values are used in the definition of different sperm movement patterns, it is understood that these values are secondary indicators of aspects of flagellar movement when the spermatozoa are analysed in seminal plasma.

The relationship between flagellar movement and head movement may be more complex for human spermatozoa in capacitating medium, although this has not been studied in detail. For example, it has been shown that human spermatozoa, when incubated under capacitating conditions, exhibit different motility patterns such as ‘hyperactivated’ [both progressive (‘transitional’) and non-progressive (‘star-spin’ or ‘thrashing’) head movement patterns], ‘freeze-flex’ and ‘forward progressive’ (Burkman, 1984; Robertson et al., 1988), and that they are able to switch between these motility patterns (Mortimer and Swan, 1995a). Despite obvious differences in head and flagellar movement patterns between the fairly regular seminal spermatozoa and the more erratic capacitating spermatozoa, use of the original kinematic values has persisted in the description of hyperactivated motility. This has contributed to some confusion over track classification, with the result that investigators may not always be certain that a trajectory classified in one laboratory as hyperactivated would have the same classification in a different laboratory.

One way to overcome this confusion would be to derive a new set of kinematic values for use with capacitating human spermatozoa. Because sperm head movement is dictated by flagellar movement patterns, this would require an understanding of the relationship between the new centroid kinematic values and flagellar movement parameters. The first step in this process is to investigate quantitative differences in flagellar
movement patterns between hyperactivated and non-hyperacti-
vated spermatozoa. This would allow the comparison of any new
centroid-based kinematic values with features of flagellar
movement of capacitating human spermatozoa, thereby ensur-
ing the relevance of these new values. This approach is the
same as that used in the derivation of the current kinematic
measures (David et al., 1981).

Few detailed quantitative studies of the flagellar movement
of capacitating human spermatozoa have been published, and
those which are available have studied flagellar movement
either at image sampling frequencies of \(\approx 60\) Hz (Burkman,
1984; Ishijima et al., 1986; Morales et al., 1988) or with
shallow preparations (Hoshi et al., 1988). While these image
sampling frequencies are sufficient for imaging and tracking
sperm head movement, and are therefore suitable for CASA,
it is possible that they may be too low for flagellar analysis
and quantitative information about flagellar wave properties
may be lost. Therefore, while it is not practical to analyse
sperm movement routinely at high image sampling frequencies,
these image sampling frequencies are required for research
into flagellar movement patterns, especially of capacitating
spermatozoa.

Therefore, the aim of this study was to determine whether
quantitative differences exist between the flagellar movement
patterns of hyperactivated and non-hyperactivated capacitating
human spermatozoa when analysed at image sampling frequen-
cies \(>60\) Hz, with the long-term goal of using these values in
the determination of the relevance to flagellar movement of
any new, centroid-based kinematic measures for capacitating
human spermatozoa.

Materials and methods

Sperm preparation

Semen was collected by masturbation from a normal, fertile volunteer
following a requested period of 3 days sexual abstinence. After
liquefaction was completed (30 min at 37°C), 0.25 ml volumes of
spermatozoa were layered under 0.8 ml volumes of human tubal fluid
(HTF) medium (Quinn et al., 1985) supplemented with 30 mg/ml human
serum albumin (HSA; Fraction V; Irvine Scientific, Santa Ana, CA,
USA) in small plastic, round-bottomed tubes (#2003; Falcon Plastics,
Oxnard, CA, USA). Motile spermatozoa were allowed to swim up
into the medium during a 40 min incubation at 37°C. The upper
0.5 ml of medium were collected from each tube, taking care to
avoid contamination of the preparation with seminal plasma. The
supernatants were combined in a fresh tube and incubated for a
further 30 min at 37°C.

Videomicrography

Sperm movement was recorded in 50 \(\mu\)m-deep chambers with a fixed
coverslip (Micro-Cell HAC, Conception Technologies, La Jolla, CA,
USA). Approximately 7 \(\mu\)l of the sperm preparation were loaded
into each chamber, which had been pre-equilibrated to 37°C on a
warming stage.

Video recordings (NTSC format) were made using an NAC HSV-
200 camera and video recorder system attached to a Reichert Univar
microscope. A \(\times 20\) positive low phase-contrast objective was used,
with a \(\times 2.5\) camera ocular and a \(\times 1.5\) intermediate. A time/date
generator was wired in series, and a 0.001 s time code was embedded
onto the video tape during recording.

The final magnifications were calibrated from recordings of a
Makler chamber (Seishi Medical Instruments, Haifa, Israel). When the
video tapes were replayed on a Panasonic TC61A61 TV monitor
(Matsushita Electric Co., Penrith, NSW, Australia) using a Panasonic
NV-F66A VCR (200 Hz image sampling frequency; Matsushita) for
centroid movement analysis, the final magnification was \(\times 1900\). When the video tapes were replayed using a Sony SLV-X811AS VCR
and an Orion 9025 21E monitor (Sony Australia, North Ryde, NSW,
Australia) for the 100 Hz manual flagellar analysis study, the final
magnification was \(\times 1495\).

Sperm movement analysis

Centroid analysis

To confirm the subjective classification of tracks as hyperactivated
or non-hyperactivated, their centroid trajectories were reconstructed
and analysed manually. The segments of the trajectories which were
used for flagellar analysis were identified and 1 s of centroid
movement, which included these segments, plotted as described
previously (Mortimer and Swan, 1995b). Because there are no
published definitions for the classification of 200 Hz centroid traces
as hyperactivated, every third point on the trajectory was considered,
to approximate the flagellum (Mortimer et al., 1988). Each point on
the 67 Hz tracks was given an \((x,y)\) coordinate, determined by placing
the trajectory over mm graph paper, and the coordinates were entered
into spreadsheets. The VCL, linearity (LIN) and ALH were determined
for each track, and compared with published definitions for hyperactiv-
iton analysed by manual methods at \(60\) Hz (i.e. VCL \(>180\) \(\mu\)m/s
and LIN \(\leq 45\%\) and ALHmean \(>6.0\) \(\mu\)m or ALHmax \(>10.0\) \(\mu\)m;
Mortimer and Swan, 1995b).

Semi-automated flagellar analysis

SIAM FLAG (LHESA Electronic, Cergy Pontoise, France), a semi-
automated flagellar movement analysis system, was used in the first
portion of the study. A total of 30 consecutive video images at 200 Hz
were analysed for each track. When a spermatozoon of interest was
identified, the cursor cross-hairs were placed over its video image.
The program required the manual identification of the sperm head
top and head–midpiece junction prior to placing the cursor cross-
hairs over appropriate locations on the periphery of the sperm head
and clicking with the mouse. This defined the longitudinal axis of
the head used during the track’s analysis. The computer then identified
the flagellum, and displayed the spline curve it was using to
approximate the flagellum (Schoevaert et al., 1988, 1990). It was
possible at this stage to correct any errors made by the computer.
For example, if a piece of debris had been assumed by the computer
program to be a part of the flagellum, part of the computer-generated
curve could be erased and re-drawn manually by tracing over the
flagellar image and placing consecutive dots along the tail using the
computer mouse. After the flagellum had been imaged satisfactorily,
the image was stored and the next frame analysed. When all 30
images for a spermatozoon had been stored, the track was scored
and flagellar movement analysis performed.

In the present study only the flagellar beat envelopes were analysed
using this system. The beat envelopes were constructed by aligning
all of the track’s images using the head axis, thereby removing the
forward movement component and allowing visualization of the
deviation of the flagellum from the longitudinal axis of the spermato-
zoa. Print-outs of the beat envelopes were produced for each
spermatozoon studied, and the maximum angle of deviation of the
flagellum about the midline (the flagellar beat angle; FBA) was
determined manually using a protractor (Figure 1).

Manual flagellar reconstruction and analysis

The same sequences included in the SIAM FLAG analysis were
identified using the video tape time-base. When a spermatozoon of
interest was identified, the tape was advanced to the same starting point as for the semi-automated analysis. A piece of tape with a cross drawn on it was attached to the monitor’s screen close to the spermatozoon of interest, but not so close that it covered any of the cell during the analysis sequence. The head outline and tail were traced onto overhead projector film sheets using fine-tipped marker pens. The cross was traced with every image, giving the position and orientation of the spermatozoon with respect to it.

Because of the limitations of resolution using video tape, it was not always possible to identify the midpiece of a spermatozoon. So the tracing was only of the head and tail, and analyses were started at the apparent junction between the head and tail of the spermatozoon (i.e. the neck). In addition, it was not possible to differentiate principal and reverse bends using these traces because the image was not always clear enough to differentiate head rotation and there were no obvious cellular points of reference.

The flagellar traces were photocopied using a machine known not to distort the copied image, and the copies were compared with the originals and found to be accurate. Manual flagellar analysis was made using the photocopied images.

Calculation of the peak of the flagellar wave
The peak of a wave was determined by the geometric bisector method. First, the tangents to the wave were drawn. Then, using a drawing compass with the point placed on the point of intersection of the tangents, arcs were drawn onto the tangents. With the drawing compass set to the same distance, its point was placed on each intersection of the tangent and arc and another arc drawn below the wave peak. The intersection of these arcs and the intersection of the tangents were joined to give the bisector of the wave (David et al., 1981). The distance from the neck of the spermatozoon to the bisector was measured in millimetres by tracing the flagellum with a curvimeter (Model No. 54M; Burnat, Paris, France). The millimetre distance was converted to microns by dividing by the magnification correction factor (final magnification/100) (Figure 2).

Calculation of the velocity of propagation of the flagellar wave
The peak of the wave was followed along the flagellum in consecutive images and the velocity determined by the change in distance (∆ distance) over time (since 0.01 s elapsed between successive images).

Calculation of the diameter of the flagellar wave
A series of circles of diameters from 10 to 50 mm (increasing in 1 mm increments) was drawn using ‘CorelDRAW!’ (Version 3.0; Corel Corporation, Ottawa, Ontario, Canada) and printed onto a single sheet of paper. The circles were photocopied onto overhead transparency film, and the accuracy of the copy checked by placing the transparency over the original. The accuracy of the diameters was checked using a mm rule.

The diameter of the flagellar waves was estimated by placing the transparency over the flagellar trace and finding the circle that best fitted the flagellar bend. The diameter was then converted to μm by dividing by the magnification correction factor (final magnification/100) for the particular flagellar trace (Figure 2).

A large-diameter flagellar wave was one with a low degree of flagellar deformation, while a small-diameter flagellar wave indicated a high degree of flagellar deformation, i.e. the flagellum had a more acute bend.

Calculation of the amplitude of flagellar waves
The amplitude of a wave is the perpendicular distance between the point of inflexion and the peak. The point of inflexion of the flagellar waves could not be determined accurately, so the total perpendicular distance between a consecutive peak and trough was found, and the distance halved to give the amplitude (in mm). The amplitude was converted to μm by dividing it by the magnification correction factor (final magnification/100) for the particular flagellar trace (Figure 2).

Calculation of flagellar beat frequency (FBF)
The FBF was estimated for each spermatozoon by determining the number of initiations per second. The flagellar traces were examined and the initiation of a wave was defined as the first image showing a flagellar bend which was subsequently propagated. The number of initiations was counted, and the frequency converted to Hz (cycles/s).

Statistics
Receiver Operating Characteristic (ROC) curve analyses were used to determine hyperactivation thresholds. When the values were normally distributed, comparisons were made using unpaired t-tests, and when they were not normally distributed, comparisons were made using unpaired Wilcoxon analyses. All statistical analyses were performed using MedCalc (MedCalc Software, Mariakerke, Belgium).

Results
Trajectory classification
Centroid analysis of the tracks confirmed the subjective classification of the spermatozoa as hyperactivated or non-hyperactivated. The objective classification of these tracks meant that
they could be considered to be representative of each motility type.

**Flagellar beat angle**

A total of 50 tracks at 200 Hz were analysed using SIAM FLAG. The classification of 24 tracks as hyperactivated and 26 as non-hyperactivated was made according to previously established criteria (Mortimer and Swan, 1995b). Only progressive (transition phase) hyperactivated tracks were used in this study, because it was not possible to keep the flagella of the ‘star-spin’ hyperactivated cells in focus for a sufficient length of time to perform meaningful analyses.

The FBA about the head–midpiece junction was significantly greater ($P < 0.001$) for hyperactivated spermatozoa (median 175°, range 96–242°) than for non-hyperactivated spermatozoa (median 72°, range 55–87°) (Figure 3). ROC curve analysis of the FBA gave 100% sensitivity and 100% specificity for discrimination between hyperactivated and non-hyperactivated flagellar movement at a threshold of 87° (Figure 3).

These results indicated that the flagellar beat patterns were significantly different between hyperactivated and non-hyperactivated spermatozoa, with the hyperactivated flagellar wave having a much wider beat envelope.

**Manual flagellar analysis**

The same spermatozoa as used in the FBA analysis were identified and their flagellar movements reconstructed manually. Flagellar waves were identified and only those where the whole wave could be followed were included, giving a total of 19 non-hyperactivated and 21 hyperactivated spermatozoa. The number of waves analysed ranged from one to five per spermatozoon, with a total of 47 waves for the hyperactivated and 48 for the non-hyperactivated spermatozoa.
Figure 5. Velocity of wave propagation versus distance of the wave peak along the flagellum. Values shown are means ± 2 SEM (95% confidence interval); solid line = non-hyperactivated; dashed line = hyperactivated. One-way analyses of variance revealed a significant effect of distance on the velocity of the flagellar wave for both hyperactivated and non-hyperactivated spermatozoa. The non-hyperactivated wave had a significantly higher velocity than the hyperactivated wave (t = 9.643, P < 0.0001).

Figure 7. Amplitude versus distance of the wave peak along the flagellum. Values shown are means ± 2 SEM (95% confidence interval); solid line = non-hyperactivated; dashed line = hyperactivated. The hyperactivated waves had significantly higher amplitudes (Z = –9.03, P < 0.0001), which increased with propagation. In contrast, the non-hyperactivated waves increased in amplitude only slightly with propagation.

In summary, analysis of the flagellar traces revealed that:

(i) the hyperactivated wave was initiated at the same position along the flagellum as the non-hyperactivated wave; (ii) the hyperactivated wave was propagated more slowly than the non-hyperactivated wave, presumably because of the increase in amplitude that was associated with propagation, as well as the development of a wave with a very small radius; (iii) the diameter of the flagellar wave was lower for hyperactivated spermatozoa, although it decreased more sharply with propagation in the non-hyperactivated spermatozoa, again presumably because of the differences in amplitude and flagellar curvature observed upon initiation and propagation of the wave.
Flagellar movement of human spermatozoa

Flagellar movement of human spermatozoa has been studied to understand the mechanisms of sperm motility. Early analyses were limited by the image sampling frequency of standard video cameras, which was typically 15 Hz. Quantitative observations of flagellar beat width and frequency reported in these studies were similar to the image sampling frequency and hence could not be relied upon. Additionally, these studies did not measure the velocity of the flagellar wave or changes in its propagation. Morales et al. (1988) used a higher image sampling frequency (60 Hz) for the analysis of capacitating human sperm populations, but no attempt was made to derive a set of quantitative definitions for hyperactivated motility. Instead, general conclusions were based on differences observed between spermatozoa with normal and abnormal head morphologies from a series of fertile donors and infertile patients.

Figure 8. Comparison of flagellar beat frequencies for hyperactivated (HA) and non-hyperactivated spermatozoa. The dotted line is the hyperactivation threshold determined by ROC curve analysis (HA > 29.4 Hz). The non-hyperactivated spermatozoa had significantly higher flagellar beat frequencies than the hyperactivated spermatozoa (t = 10.247, P < 0.0001).

Discussion

While centroid movement analysis is the most commonly used method for the classification of tracks as hyperactivated, it is the flagellar movement that changes with hyperactivation and the centroid movement pattern is a secondary indicator of this change. Manual analysis of flagellar movement is not used in population studies of sperm movement because high-speed imaging systems are required and a higher magnification is necessary for the analysis of flagellar movement than for centroid movement, meaning that fewer cells are visible per field for analysis. However, when new centroid movement criteria are proposed, the flagellar movement patterns should be investigated in relation to them, to ensure that the centroid parameters reflect genuine flagellar phenomena. It is believed that this approach would reduce the incidence of incorrect classification of movement patterns.

Previous studies which resulted in the development of centroid kinematic values presently employed for human sperm movement analysis used this approach. Both flagellar and head movements of spermatozoa were analysed, with the kinematic values developed for sperm head movement having been found to reflect sperm flagellar movement (David et al., 1981; Serres et al., 1984). However, these studies considered only sperm movement in seminal plasma. It is known that sperm movement in semen is quite regular, with the sperm head movement mimicking flagellar beat patterns. The movement patterns of capacitating human spermatozoa are quite different from those of seminal spermatozoa, with a range of movement patterns observed, such as hyperactivated motility.

The flagellar movement patterns of human sperm populations in synthetic culture media have been described in previous studies, although no quantitative descriptions of hyperactivated motility have been published with analysis at high image sampling frequencies. Burkman’s (1984) description of human sperm hyperactivation included both head and flagellar movement patterns, but these analyses were performed at 15 Hz because of limitations of standard video cameras at that time. Quantitative observations of ‘flagellar beat width’ and beat frequency were reported, but the frequency values were similar to the image sampling frequency, and hence could not be relied upon. In addition, this study did not measure the velocity of the flagellar wave, nor changes in its propagation. Morales et al. (1988) used a higher image sampling frequency (60 Hz) for the analysis of capacitating human sperm populations, but no attempt was made to derive a set of quantitative definitions for hyperactivated motility, as the general conclusions drawn were based on differences observed between spermatozoa with normal and abnormal head morphologies from a series of fertile donors and infertile patients. However, they did observe that some spermatozoa exhibited increased proximal flagellar bending and amplitude with decreased FBF, and presumed that these spermatozoa were hyperactivated. A third study of hyperactivated human spermatozoa used a 200 Hz image sampling frequency, but sperm movement was recorded in a Makler chamber (Hoshi et al., 1988) which probably constrained flagellar movement. A recently published consensus between CASA users has emphasized the importance of utilizing chambers of ≥30 µm depth to allow unconstrained flagellar movement for hyperactivation analysis (ESHRE Andrology Special Interest Group, 1996). While these earlier studies contributed significantly to the general acceptance of hyperactivation in human spermatozoa, more information is required on the specific parameters of flagellar movement associated with this movement pattern, derived from high image sampling frequency analyses, before confident evaluations of new centroid-based kinematic values can be made.

In this study we identified hyperactivated and non-hyperactivated spermatozoa and confirmed their classification objectively using previously published methods of centroid analysis (Mortimer et al., 1988; Mortimer and Swan, 1995b). While the spermatozoa included in this study were obtained from a single donor, the assessment of the centroid trajectory of each spermatozoan and comparison with published hyperactivation threshold values ensured that the spermatozoa studied could be considered to be representative of the movement types, thereby allowing confidence in comparisons between flagellar movement patterns. Quantitative differences were observed in flagellar movement between these motility types. All of the parameters studied were significantly different between hyperactivated and non-hyperactivated spermatozoa, with threshold values derived for the angle of the beat envelope (≥87°) for hyperactivated spermatozoa and for the beat frequency (≥29.4 Hz for hyperactivated spermatozoa). While the beat frequency was determined using 100 Hz flagellar traces, the fact that the hyperactivation threshold value was well below the Nyquist number (where the frequency of the event must be less than half the frequency of the observations) would indicate that the value obtained was not subject to aliasing, and could therefore be applied with confidence. However, the observation that the non-hyperactivated spermatozoa had FBF above 30 Hz therefore has dramatic implications for the use of FBF or beat-cross frequency for capacitating human sperm populations at

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image sampling frequencies of ≈60 Hz. This forms the basis for a separate, ongoing study.

The amplitude of the flagellar wave was also found to be significantly different between hyperactivated and non-hyperactivated spermatozoa, as expected from the FPGA results. The non-hyperactivated wave did not increase markedly in amplitude with propagation, although the flagellum was bent more tightly with propagation (Figures 6 and 7). In contrast, the amplitude of the hyperactivated wave increased significantly with propagation up to 15 µm along the tail, accompanied by a significant decrease in the bend diameter over the same region. The bend diameter then remained essentially stable for the remainder of the wave propagation, with some increase in amplitude, although this was not significant when the 95% confidence limits were considered (Figures 6 and 7).

The hyperactivated flagellar wave was found to have a significantly lower velocity, taking >0.02 s longer to reach the same 30 µm point on the flagellum than the non-hyperactivated wave (Figure 4). It was also found by analyses of variance that the velocity of the wave changed with regard to the distance of the wave peak along the flagellum for both motility types (P = 0.001 for hyperactivated and P < 0.02 for non-hyperactivated spermatozoa). The velocity of the non-hyperactivated wave increased significantly during propagation up to 15–20 µm along the flagellum when the 95% confidence intervals were considered (Figure 5). In contrast, the hyperactivated wave velocity did not increase significantly until it reached 15–20 µm along the tail, indicating a delay in its propagation relative to the non-hyperactivated wave. This propagation delay could be attributed to the development of the high amplitude wave, requiring a great deal of flagellar deformation in the initiation phase, because the area of the sperm tail with the greatest increase in amplitude and flagellar bending corresponded to the area with the lowest velocity of wave propagation for the hyperactivated spermatozoa (i.e. <10 µm to 10–15 µm).

While watching the spermatozoa swimming in slow motion on the video replays during the flagellar reconstruction portion of this study, differences were observed in the relative stiffness of hyperactivated and non-hyperactivated flagella, with the hyperactivated spermatozoa appearing to have much more flexible tails. The biochemical and biophysical reasons behind this observation are not clear at present, although they could be related to recent observations of differences in the phosphorylation states of capacitating spermatozoa (Kopf et al., 1995).

The existence of quantitative differences in the flagellar movement patterns of hyperactivated and non-hyperactivated human spermatozoa is now established and will be taken into account in the development of new centroid-based kinematic values for human spermatozoa, providing a foundation for comparing any new values with flagellar movement characteristics. This approach has been used in a further study on the development of smoothing-independent kinematic definitions of hyperactivated motility for use with 60 Hz centroid trajectories.

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**References**


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