# CASA—Practical Aspects



SHARON T. MORTIMER

*From the Genesis Fertility Centre, Vancouver, Canada.*

When computer-aided sperm analysis (CASA) instruments were first brought into the marketplace in the mid-1980s, it was suggested that these machines could replace the laboratory andrologist because they could provide concentration and percentage motile values for a sample, as well as give population values for aspects of sperm movement (kinematics). Unfortunately, this hope was not realized when it was shown that these early-generation machines did not provide sufficiently accurate results to act as semen analyzers (Mortimer and Mortimer, 1988, 1998). Despite the fact that CASA instruments at that time were really only designed, and therefore only suitable, for research applications, the disappointment in their apparent failure to perform as purported tarnished the reputation of CASA and delayed development of the field. It is only in relatively recent times that there has been a positive and concerted action to define the role of CASA in both the clinical andrology laboratory and in the research laboratory. There have been three international consensus meetings in which aspects of the use of CASA have been discussed and guidelines published (Mortimer et al, 1995; ESHRE Andrology Special Interest Group, 1996, 1998). The aim of these meetings was to attempt to standardize the methods and applications of CASA so that there would be the highest likelihood of useful clinical and research data being generated and applied throughout the world.

The importance of such an aim cannot be overestimated in the use of CASA. CASA is potentially a very powerful research and clinical tool. Indeed, its inclusion in reproductive toxicology studies indicates its relevance (Slott et al, 1993, 1995, 1997), but its features and limitations must be understood quite clearly before attempting to make clinical diagnoses on the basis of its results (ESHRE Andrology Special Interest Group, 1998).

## Sperm Movement

There is a great deal of variability in the movement patterns shown by spermatozoa, depending upon a variety of external and intrinsic factors. Also, it would be very unusual for all of the cells in any sperm population to be showing the same movement patterns at any one time. It is necessary to have a good understanding of how a sperm cell moves before the relevance of CASA can be fully appreciated. A number of reviews on sperm movement have been published, and the reader is encouraged to consult them (eg, Gagnon, 1995; Mortimer, 1997). In the simplest terms, the sperm cell is made up of a head, midpiece, and tail, and in missile terminology, these could be made analogous to the payload and its delivery system. The head contains the "payload," that is, chromatin that has been stabilized by extensive cross-linking and that remains inert until fertilization has occurred. The head membranes are highly specialized and undergo a series of changes during passage through the female reproductive tract. The "delivery system" is composed of the midpiece, which contains spirally arranged mitochondria that produce the energy required for sperm movement and the flagellum that propagates the propulsive waves initiated at the midpiece. The flagellum contains 9 microtubule doublets arranged around a central pair of microtubules (Figure 1). Extending from each doublet towards the next doublet are a series of arms composed of dynein (an ATPase). Under the influence of ATP, the dynein arms move and touch the next microtubule doublet in the circle and move the first doublet in a headwise direction. The dynein arms then release the second doublet. In turn, the second doublet's dynein arms reach out and touch the third doublet in the series, causing the second doublet to move forward. This cycle continues until the wave has been sent in a helical direction along the flagellum. The wave is seen as a flattened helical beat of the tail, although it may not appear so in two dimensions. As the wave travels along the tail, the spermatozoon is propelled forward, and the sperm head is forced to rotate along the axis of the direction of travel. To continue with the earlier analogy, the head has no influence on the direction of movement because as the payload, it has no navigational influence but simply moves in response to the flagellum. So, if in the initiation phase the flagellar waves are near to the head and small in amplitude, then the sperm head will have a small lateral movement and a more pronounced forward movement. If the flagellar waves are of a high amplitude before they are propagated, then the head will have a much wider lateral movement, and this will seem more pronounced than the forward movement of the cell. It has been shown that the movement pattern of spermatozoa is influenced by their external environment. For example, spermatozoa in seminal plasma have lower progressive velocities and less lateral head movement than spermatozoa from the same ejaculate that have

Correspondence to: Sharon T. Mortimer, Genesis Fertility Centre, 555 W 12th Ave, Suite 550, Vancouver, BC V5Z 3X7, Canada (email: labdir@genesis-fertility.com).

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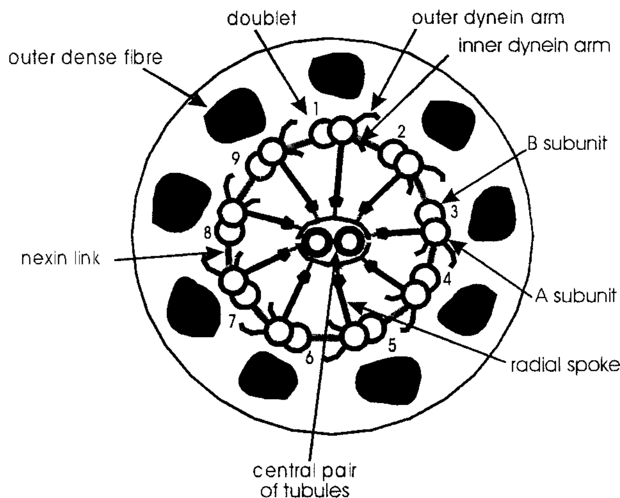


Figure 1. Diagrammatic cross-section of a mammalian sperm tail in the proximal region of the principal piece (Mortimer, 1997).

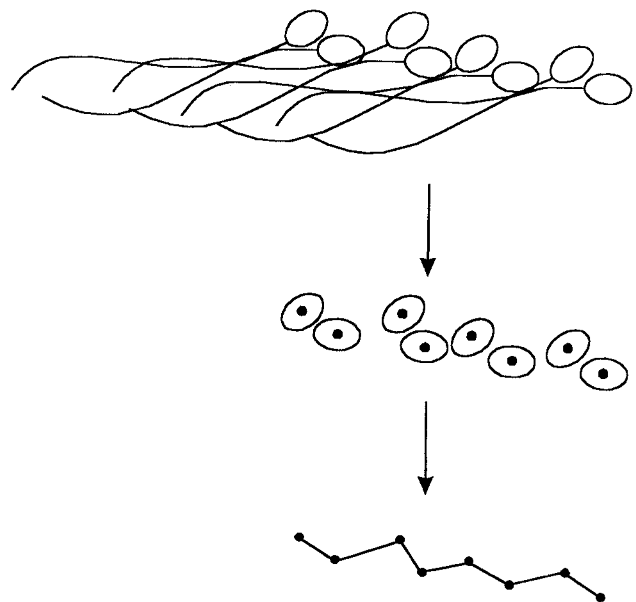

Figure 2. Reconstruction of a trajectory. A point of reference, such as the center of the sperm head (the centroid) is followed through a sequence of images of the spermatozoon. The points are then joined to give a trajectory reconstruction.

been incubated under capacitating (eg, fertilization-promoting) conditions (Serres et al, 1984).

But if the flagellum is the part of the spermatozoon that causes motility, why consider how the head is moving, rather than concentrating on the flagellar movement patterns? The reason is a practical one—it is technically much easier to follow the head than it is to follow the tail. The frequency of the flagellar beats is very high (up to 80 beats per second for washed human spermatozoa [Mortimer ST et al, 1997]), so there would need to be at least 200 observations per second for each spermatozoon to ensure that the flagellar beat pattern was being followed correctly. Because standard video systems analyze at 25–60 images per second, the frequency of sampling then would be too low, the consequence being that the tail would appear as a smear when freeze-frame analysis was used. In contrast, because the head is not moving as fast as the tail, it is possible to obtain relatively clear images of the head using conventional video technology. Because the pattern of sperm head movement is directed by the flagellar beat pattern, there is a relationship between the two, and this can be exploited in the determination of differences between sperm movement patterns under different circumstances. The reasons why this approach is important in clinical andrology and ART laboratories, as well as in research, are discussed below.

## How CASA works

To understand the most appropriate applications for CASA, the first step is to understand how CASA works—how does the microscope image of a group of swimming spermatozoa get translated into the series of measurements and numbers given by a CASA instrument? The review by Boyers et al (1989) gives a very clear presentation of the steps involved, and the reader is encouraged to consult that article for an in-depth explanation. The common components of CASA instruments are a video camera, a video-frame grabber card, and a computer. The computer software is used to identify and follow all of the spermatozoa in the video images and to perform all of the data calculations.

First, the image of the microscope field is sent from the camera and is converted into a digital image. The easiest way for the machine to perceive spermatozoa is to use a dark-field or a negative-high–phase contrast image, which gives white sperm heads on a dark background. The brightness of the head image stays fairly consistent even as the spermatozoa move because the rotation of their heads does not change the intensity of the white image. The image of each sperm head is then digitized, with the computer determining the number of picture elements (pixels) the sperm head covers (Figure 2). There is a range of pixel numbers that is acceptable for a sperm head, given the expected minimum and maximum size for the species, and the computer will recognize an object that falls within the expected range as a sperm head. Historically, CASA instruments could not differentiate between a sperm head and debris; thus, if the piece of debris was in the size range expected for a sperm head, this would be a source for error. The Stromberg-Mika CASA system addressed this problem by requiring an attached tail before a sperm head was identified positively (Neuwinger et al, 1990; Wijchman et al, 1995). In an alternative approach, the Hamilton Thorne system now uses

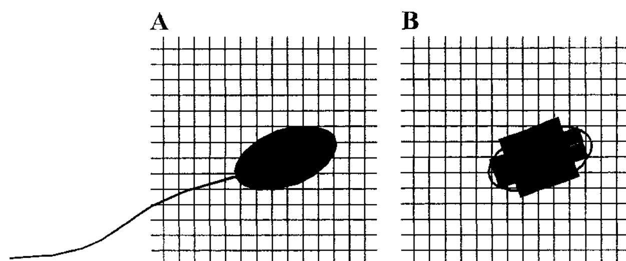


Figure 3. Digitization of the image of a sperm head. **(Panel A)**, a spermatozoon image with the overlaying picture elements (pixels) that are used to make up the video image. **(Panel B)**, how the computer "sees" the sperm head following digitization.

the IDENT system, in which Hoechst dye binds to the DNA in sperm heads, which then fluoresce and are recognized by the computer (Zinaman et al, 1996). After a sperm head has been identified, its (x,y) coordinate position on the plane of the microscope field is calculated by the computer. This point is determined using either the center of the sperm head (termed the *centroid*) or the brightest point on the sperm head as the reference point (Figure 2).

After all of the sperm heads in a single field or frame have been identified and recorded as computer data, the next field or frame is analyzed, and the digitized sperm head images are tracked by the computer (Figure 3). The computer searches for the consecutive image of a sperm head within a zone of probability, which is a circle of a particular radius around the sperm head (Figure 4). The radius is determined by the maximum distance a spermatozoon would be expected to travel within the time period. This distance is often set by the user and differs

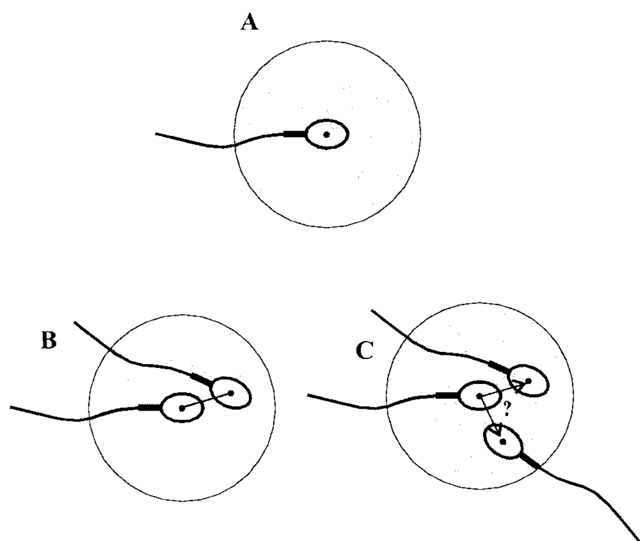


Figure 4. Identification of the spermatozoon and its zona of probability **(A)**, indicating where the spermatozoon is likely to be found in the next video image **(B)**. Complications occur when more than one sperm head is located within the zone of probability in the subsequent image **(C)**.

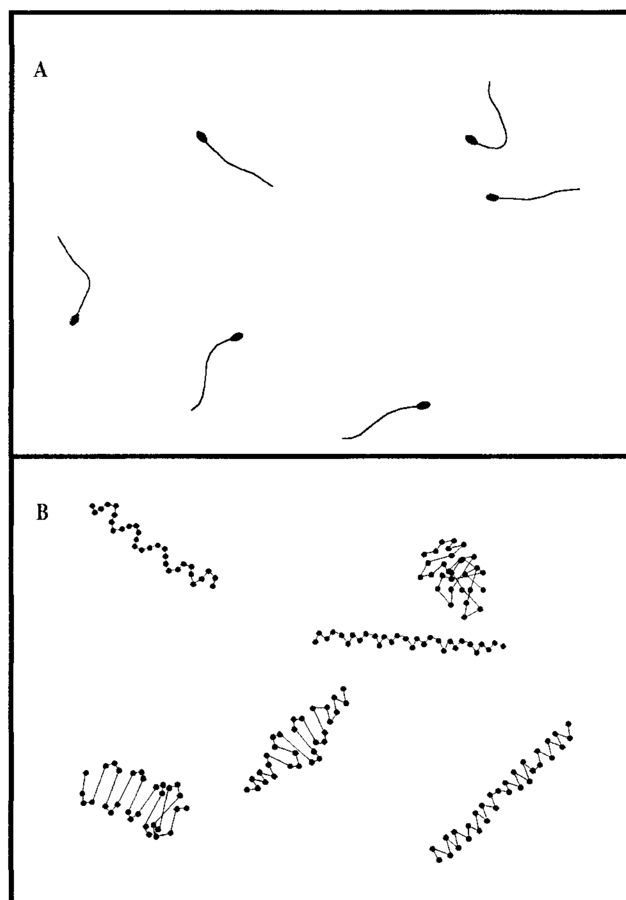


Figure 5. CASA digitization and reconstruction of sperm tracks. All of the spermatozoa in the field of view are digitized **(A)**, and their trajectories are reconstructed simultaneously over 0.5 s of movement **(B)**. Note the differences in the trajectory shapes and the relationship to differences in the flagellar bending.

depending upon whether the spermatozoa are in semen or in culture medium. After the sequential image of the sperm head has been located, its (x,y) coordinates are calculated, and the next video field or frame is analyzed, until the time period is completed. For each spermatozoon, the trajectory of the reference point on the sperm head is reconstructed (Figure 5), and a series of kinematic values are calculated. Kinematics are "time-varying geometric aspects of motion" (Drobnis et al, 1988b) and are used to differentiate sperm movement patterns. The relevance of the different sperm movement patterns in the diagnosis of sperm function is discussed in detail below. When using CASA, all of the spermatozoa in the field of view have their trajectories reconstructed simultaneously. That is, all of the spermatozoa in a field are identified before the next image in the sequence is analyzed. It follows then that if too many spermatozoa are in the field of view, it may not be possible for the instrument to reconstruct their trajectories accurately. If spermatozoa collide with each other, their kinematics are momentarily al-

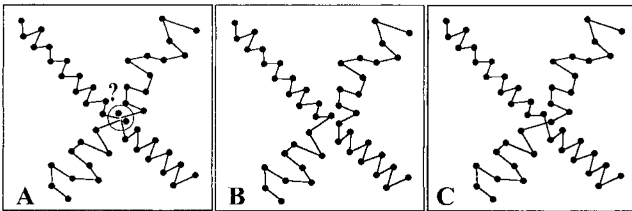


Figure 6. Trajectory reconstruction when two spermatozoa share a zone of probability **(A)**. In these cases, the CASA instrument will either truncate the sperm tracks or determine which is the most likely trajectory. There is the risk that the tracks may be incorrectly reconstructed **(B)**, although vector analysis would reduce the incidence of this **(C)**.

tered, and this could affect their motility classification. Also, if the trajectories of two or more spermatozoa bring them to within each other's zone of probability, then depending upon the CASA instrument used, the trajectories may be rejected from the analysis or may be included, but with the risk of incorrect reconstruction (Figure 6). It is for these reasons that the consensus meetings on the use of CASA have recommended that sperm concentrations of $<40 \times 10^6$/mL be used (Mortimer et al, 1995b; ESHRE Andrology Special Interest Group, 1998).

The camera used to obtain the microscopic images is a video camera. The number of images per second that the camera obtains depends upon the picture standard used. For example, in some countries, the phase alternating line (PAL) standard is used, whereas in others, it is the National Television Standards Committee standard (NTSC). For both of these video standards, the video camera records 2 video fields, 1 field composed of the odd-numbered lines, or rasters, that make up the video picture, and the other field composed of the even-numbered lines. These two fields are usually combined to provide a "frame." The PAL system gives 25 frames, or 50 fields, per second, whereas the NTSC system gives 30 frames, or 60 fields, per second. In the past, only 0.25–0.5 seconds' movement was analyzed by CASA, but with some instruments, such as the Hobson Sperm Tracker and the Hamilton Thorne IVOS, it is now possible to follow spermatozoa for extended periods. The video system used, therefore, will have an effect on the number of images of a sperm's trajectory analyzed within a set time period. The shape of the trajectory is altered quite considerably by the number of images taken per second, so the same track can appear relatively simple when reconstructed at 30 images per second, but complex when reconstructed at 60 images per second (Figure 7). This effect has been shown to alter the kinematic values for a trajectory (Mortimer et al, 1988; Mortimer and Swan 1999b).

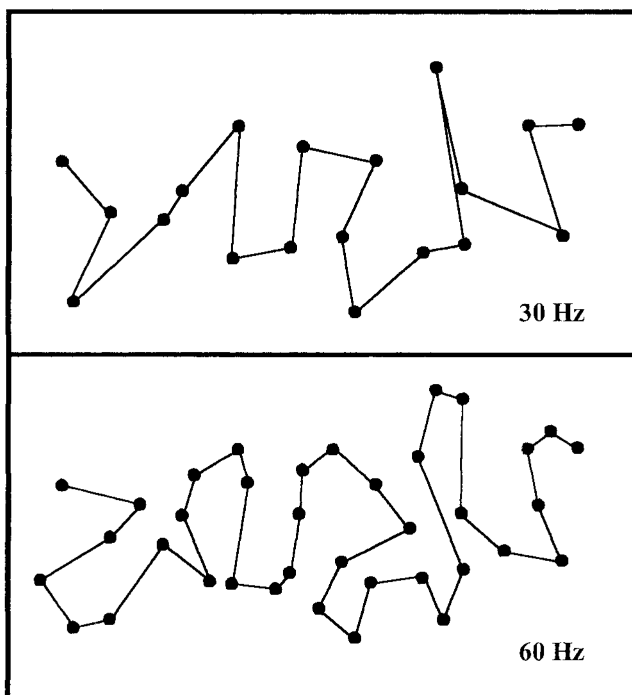


Figure 7. The same trajectory reconstructed at 30 and 60 Hz. Increasing image sampling frequency results in more track information being available and therefore affects the kinematics.

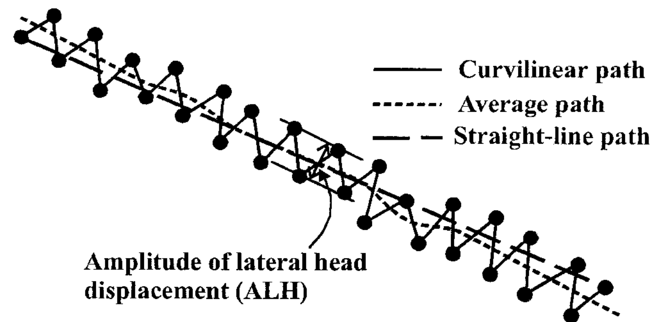


Figure 8. Determination of kinematic values. The velocity values are determined as the length of the relevant path over time. The values LIN, STR, and WOB are determined as ratios of the velocity values. Amplitude of lateral head displacement (ALH) is the total width of the sperm head trajectory. See text for details of calculation and explanation of abbreviations.

## Quantitation of Sperm Movement

The kinematic values determined for each spermatozoon cover the velocity of movement, the width of the sperm head's trajectory, and frequency of the change in direction of the sperm head (David et al, 1981; Serres et al, 1984). The derivation of each of the values to be discussed has been covered in detail elsewhere (Mortimer, 1997) and so will not be covered here.

The velocity values that are determined are the curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP; Figure 8). All of these values

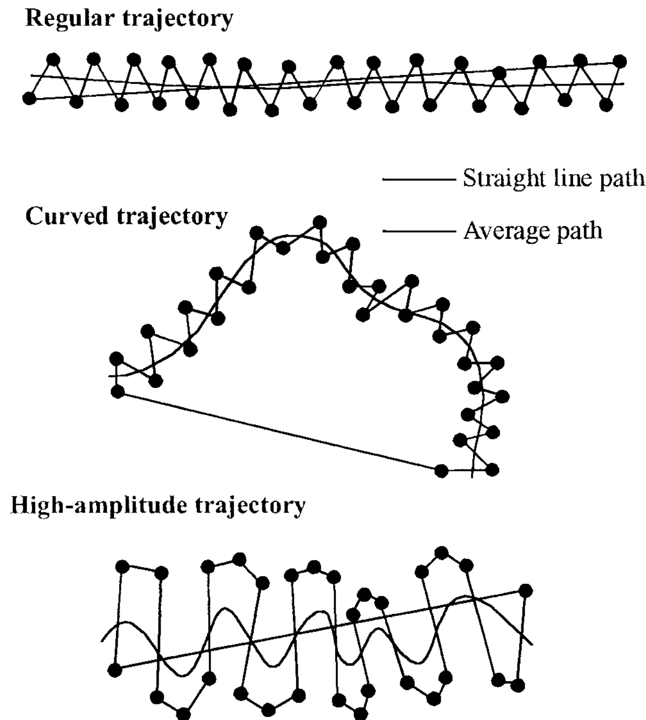


Figure 9. Relationship between straight-line and average paths for different trajectory types. For a regular trajectory, the straight-line and average paths are of similar lengths. For curved and high-amplitude trajectories, the straight-line path is significantly shorter than the average path.

are presented to 1 decimal place in units of μm/s (Mortimer, 1990). The VCL refers to the total distance that the sperm head covers in the observation period and is always the highest of the 3 velocity values. The VSL is determined from the straight-line distance between the first and last points of the trajectory and gives the net space gain in the observation period. This is always the lowest of the 3 velocity values for any spermatozoon. The VAP is the distance the spermatozoon has traveled in the average direction of movement in the observation period. This is conceptually the most difficult velocity value to understand because it might appear that it should be similar to the VSL. In cases in which the sperm head's trajectory is very regular and linear, with very little lateral movement, then the VAP is almost the same as the VSL. However, with irregular trajectories, such as those that are not linear, or where there is a high degree of lateral deviation of the head about the direction of movement, then the VAP will be much higher than the VSL (Figure 9). The average path is determined by CASA using track smoothing. In its simplest form, this means that for each point along the sperm trajectory, an average is taken of its (x,y) coordinates as well as those of the two track points on either side of it. This gives a smoothed (x,y) coordinate for that track point. The procedure is repeated for each point along the track, resulting in the construction of a smoothed average path. The VAP is the length of this path over the observation period.

Because the trajectory shape influences the velocity values, the velocity values are also compared. The ratios of the three velocities are linearity (LIN), straightness (STR), and wobble (WOB) and are derived as follows:

$$\mathrm{LIN} = (\mathrm{VSL}/\mathrm{VCL}) \times 100$$

$$\mathrm{STR} = (\mathrm{VSL}/\mathrm{VAP}) \times 100$$

$$\mathrm{WOB} = (\mathrm{VAP}/\mathrm{VCL}) \times 100$$

These percentage values are expressed as integers (Mortimer, 1990).

The amplitude of lateral head displacement (ALH) is the width of the lateral movement of the sperm head. It is calculated as the total width of the head trajectory (rather than half the width, which is the amplitude value used in physics and mathematics) and is expressed in micrometers (David et al, 1981). Its derivation by CASA relies upon the smoothed path determined for the VAP calculation. The distance from a track point to its smoothed point is called a *riser* (Boyers et al, 1989). For a regular trajectory, the riser distance increases to a maximum value, then decreases, then increases again, following the beat pattern. The local maxima in the riser values is half the ALH, so the ALH is determined by doubling the riser distance. Of course, this relationship is not so close for irregular trajectories, but it is still used. There are 2 possible ways of reporting ALH, either the ALHmean or ALHmax. The ALHmean is the mean of a set number of ALH readings along the trajectory, whereas ALHmax is the maximum ALH value for the trajectory segment analyzed. It is helpful to know which ALH your CASA instrument uses; for example, the Hamilton Thorne instruments all use ALHmax.

The beat-cross frequency (BCF) is the number of times the sperm head crosses the direction of movement, and this is related to the development of another flagellar wave (Serres et al, 1984). The BCF is expressed in hertz and is calculated by counting the number of times the curvilinear path crosses the average path per second. The BCF is a useful value in the estimation of gross changes in the flagellar beat pattern, but it may be hampered by the number of observations that can be made per second by CASA. If there are more beats per second than there are images per second, then the BCF will be underestimated (Mortimer and Swan, 1999b).

Recently, a new series of kinematic values has been published for the analysis of the movement patterns of capacitating human spermatozoa (Mortimer et al, 1996; Mortimer and Swan, 1999a). These values were developed as a means of investigating whether CASA results could be standardized internationally. At present, the differences in image-sampling frequencies used for track re-

construction, as well as the differences in the algorithms used by different CASA instruments for the derivation of the average path and all associated kinematic values, mean that there is the potential for the same trajectory to have very different kinematic values when analyzed using different CASA instruments. The proposed values do not rely upon the derivation of an average path for their determination, and so the effect of the different algorithms should be removed. Although most of these values have yet to be tested independently, the fractal dimension has been shown to be a useful kinematic value in several studies (Davis and Siemers, 1995; Mortimer et al, 1996; Mortimer 1998).

The term *fractal* was coined by Benoit Mandelbrot and is a contraction of *fractional* (Mandelbrot, 1983). The fractal dimension is an expression of the complexity of a line and is related to the amount of space the line takes on a plane. Geometrically, a straight line has a single dimension (length), and a plane has two dimensions (length and breadth). However, if the line is not straight, then it takes on the "dimension" of breadth because it is covering more of the plane, but it can't be considered to have 2 dimensions because it is still a line. The fractal dimension of a line is therefore an indication of how much it meanders, with a fairly straight line having a fractal dimension of, say, 1.1, whereas a very complex line will have a fractal dimension of, say, 1.8. In kinematics, the fractal dimension of a trajectory has been shown to be inversely related to LIN, but unlike LIN, it also includes a consideration of the distance traveled (Mortimer et al, 1996).

Irrespective of the methods used, CASA determines the kinematic values for each spermatozoon, and combinations of these values can be used to classify its movement pattern.

## Sperm Movement Analysis and Sperm Function

It is important to understand that the use of population averages of kinematic values, as given by many CASA instruments, is not going to allow any statistically predictive power. As mentioned earlier, there is a range of movement patterns that will be seen in any sperm population, and certainly in the human, it would be extremely unusual for all of the spermatozoa in a preparation to have the same velocity and ALH values. In the most recent consensus article with regard to the use of CASA (ESHRE Andrology Special Interest Group, 1998), it was agreed that studies that have only reported population changes in kinematic values should not be published because they do not provide any useful information. The approach of looking only at population averages is analogous to comparing the average values for all of the blood counts done in 2 different hematology laboratories and looking for evidence of an increased incidence of anemia. It just doesn't make sense, and it doesn't give any indication of the proportion of normal versus abnormal results in the populations tested. For meaningful sperm movement analysis, we need to first identify a range of normal kinematic values for the movement pattern of interest and then determine the proportion of spermatozoa in each preparation which meet these criteria.

One well-documented application of this approach is in the estimation of the proportion of mucus-penetrating spermatozoa in a human sperm population. It was found that spermatozoa that were able to penetrate periovulatory cervical mucus had a similar group of kinematic properties, for instance, VAP $\pm$ 25.0 μm/s AND ALH $\pm$ 4.5 μm (Serres et al, 1984; Aitken et al, 1985; Feneux et al, 1985; Mortimer et al, 1986). When this sort of definition is used, it is termed a Boolean argument, with the AND statement meaning that all of the criteria have to be met for the spermatozoon to be considered a member of the group of interest. If the proportion of spermatozoa in a semen sample that meet these criteria is determined, then an indication is given of the likelihood of the man's spermatozoa being able to penetrate his partner's cervical mucus, which is the first barrier to sperm transport in the female reproductive tract. This obviously has a great deal of relevance to the identification of potential causes of infertility, and the test itself, that is, evaluating the proportion of spermatozoa in semen that meet the mucus-penetrating criteria, can be run quite quickly using CASA, without the requirement for a sample of cervical mucus.

Another area in which kinematics has been used in the prediction of sperm function is in determining the proportion of hyperactivated spermatozoa in a suspension of spermatozoa prepared for assisted-conception procedures.

## Hyperactivated Motility

Hyperactivated motility occurs when the flagellum develops high-amplitude waves in the proximal, rather than the distal, region (Mortimer ST et al, 1997). The development of a high-amplitude bend means that the propagation of the wave is delayed and that the sperm head is moved away from the average path. When the wave is propagated, its movement away from the proximal region of the tail results in the sperm head being moved back toward the average path at a high velocity, much like a whiplash (which is how hyperactivated motility is often described). The kinematics of hyperactivated spermatozoa are very different from spermatozoa in seminal plasma and from noncapacitated spermatozoa. For human sper-

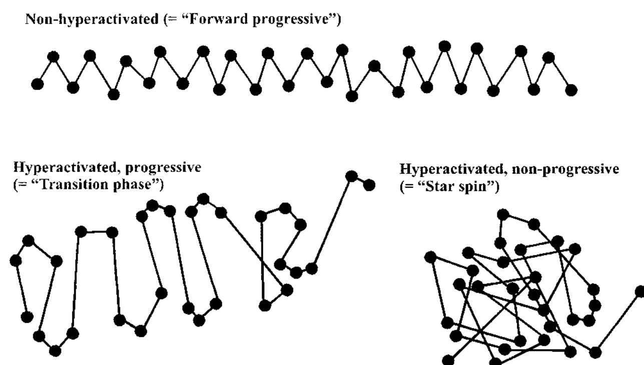


Figure 10. Motility patterns of capacitating human spermatozoa. Non-hyperactivated spermatozoa have relatively regular trajectories, with low ALH. Hyperactivated trajectories are characterized by high ALH and VCL values, with low LIN.

matozoa, there have been 2 types of hyperactivated motility described: the transition phase, or progressive hyperactivated motility, and the star-spin, or nonprogressive hyperactivated motility (Figure 10; Burkman, 1984, 1990; Robertson et al, 1988; Mortimer and Mortimer, 1990). For human spermatozoa analyzed at 60 Hz, the definition for hyperactivated motility is VCL $\pm$ 150 μm/s AND LIN $\leq$ 50% AND ALHmax $\pm$ 7.0 μm (Mortimer et al, 1998).

Human spermatozoa do not remain indefinitely in a state of hyperactivated motility but rather show a phase-switching activity between each of the 3 patterns. These switches occur apparently at random and with variable frequency (Mortimer and Swan, 1995).

Under normal circumstances, it is only spermatozoa that have been isolated from seminal plasma and incubated under capacitating conditions that exhibit hyperactivated motility (Mortimer et al, 1998), although it has been shown to occur in semen with low superoxide anion–scavenging capacity (de Lamirande and Gagnon, 1993). The motility pattern is quite striking, and the reason it occurs has been the topic of some debate, although there is evidence that it is involved in some way with the fertilization process. It has been observed to occur in in situ preparations of hamster oviduct ampullae (Yanagimachi, 1970; Katz and Yanagimachi, 1980). Also, studies of the interaction between human oviductal epithelial cells and spermatozoa *in vitro* have shown that the spermatozoa that detach from the epithelial cocultures have hyperactivated motility (Pacey et al, 1995a,b). Similarly, observations of transilluminated mouse oviducts have shown significantly more free-swimming spermatozoa (with more sharply bent flagella than the bound spermatozoa) in the ampulla than the isthmus, suggesting that hyperactivated motility may play a role in moving the spermatozoa away from the isthmic reservoir (Smith and Yanagimachi, 1991; Demott and Suarez, 1992). Rat spermatozoa have also been observed to exhibit hyperactivated motility shortly after entering the oviductal ampulla of naturally cycling rats (Shalgi and Phillips, 1988).

There are 5 suggested physiological reasons for hyperactivated motility. These are prevention of entrapment, improvement of the chances of oocyte contact, maintenance of the microenvironment, assistance in cumulus penetration, and assistance in zona penetration.

### *Prevention of Entrapment*

Because hyperactivated rabbit spermatozoa recovered from the ampulla swam in circles (Suarez et al, 1983), it was proposed that they would be less likely to be trapped in the folds and crypts of the oviductal epithelium, whereas straight-swimming spermatozoa would spiral straight into those folds and crypts.

### *Improvement of the Chances of Oocyte Contact*

Fertilization occurs in the relatively large area of the ampulla, and it is thought that the nonprogressive motion of hyperactivated spermatozoa might increase the likelihood of contact with the oocyte (Katz et al, 1978; Suarez et al, 1983).

### *Maintenance of the Microenvironment*

Because of the way the hyperactivated sperm tail moves, it has been shown to stir the surrounding fluid more than do nonhyperactivated spermatozoa (Katz and Dott, 1975). This may have the effect of ensuring a constant supply of the biochemicals required for flagellar movement (Katz et al, 1989).

### *Assistance in Cumulus Penetration*

It has been shown that only hyperactivated spermatozoa are able to enter the cumulus oophorus (Suarez et al, 1984; Cummins and Yanagimachi, 1986; Katz et al, 1986). However, the flagellar beat pattern of the spermatozoa changes to a nonhyperactivated pattern while they are in the cumulus matrix (Suarez et al, 1991), but when they leave the cumulus, they resume a hyperactivated pattern (Cummins, 1982; Drobnis et al, 1988b). Because fertilization requires the spermatozoa to traverse the cumulus matrix, these results suggest that hyperactivated motility is an integral part of the fertilization process.

### *Assistance in Zona Penetration*

The fertilizing spermatozoon forms tight chemical bonds with the zona pellucida. The force required to break these bonds and allow the spermatozoon to penetrate the zona pellucida, is around 40 μdynes, but the force generated by a free-swimming spermatozoon is around 30 μdynes (Baltz et al, 1988). However, if the sperm tail retains the hyperactivated beat pattern while the sperm head is attached to the zona pellucida, then the force generated has been calculated to be up to 2700 μdynes, which is more

than enough to break covalent bonds (Drobnis et al, 1988a). Further support for this hypothesis has been given by an experiment in which hamster spermatozoa bound to the zonae of cumulus-free oocytes had their flagellar movement inhibited by the addition of verapamil or cadmium (Stauss et al, 1995). Only 1 in 80 of these oocytes were penetrated, compared with 25 of 40 in the control treatment, indicating the importance of hyperactivated motility in zona penetration.

With the evidence gathered to date, it would appear that hyperactivated motility is an integral process in sperm transport through the female tract as well as in fertilization itself. It follows, therefore, that the assessment of the proportion of spermatozoa in a preparation that are able to express hyperactivated motility would give an indication of a critical aspect of sperm function.

## Clinical Evaluation of Hyperactivated Motility

The practical aspect of the identification of hyperactivated motility is in the prediction of the likelihood of the spermatozoa in a preparation to *fail* to fertilize an oocyte, either in vivo or in vitro. If one assumes that at least some of the hypothesized physiological reasons for hyperactivated motility are true, then the failure of spermatozoa to hyperactivate under appropriate conditions could indicate a potential for failure of fertilization. The implications of this are obvious because if such a problem could be diagnosed as part of a couple's infertility investigation work-up, it would allow their subsequent clinical therapy to be managed more effectively. For an evaluation like this, the movement patterns of several hundred spermatozoa must be studied, which is only practical using CASA.

For the evaluation, it is first necessary to isolate the motile spermatozoa from seminal plasma, in much the same way as occurs in nature at the cervix. The cervical canal contains cervical mucus, and in the periovulatory period, motile spermatozoa are able to penetrate the mucus and enter the uterus. Only spermatozoa that have good progressive motility are able to enter the mucus (as discussed above), so it acts as a barrier to spermatozoa with poor motion. In the laboratory, the best methods for the preparation of a capacitating sperm population are either by direct swim-up of semen into a culture medium overlay, or by density gradient centrifugation. A recent study has shown that density gradient centrifugation results in the selection of functionally superior spermatozoa, with significantly fewer DNA nicks, so this will probably become the method of choice (Sakkas et al, 2000). After the spermatozoa have been isolated, they must be incubated at 37°C in a $CO_2$-enriched atmosphere, in a culture medium that contains calcium and bicarbonate ions and protein, to facilitate capacitation. To estimate the proportion of hyperactivated spermatozoa in the preparation, a sample is placed in a warmed microscope chamber at least 30 μm deep (Mortimer et al, 1995b; ESHRE Andrology Special Interest Group, 1998), and the kinematics of at least 200 motile spermatozoa are determined by CASA. The depth of the chamber used is an important factor in the determination of kinematics. It has been shown that a chamber depth of 20 μm affected sperm movement, presumably by constraining the development of the flagellar beat, and altered the relative proportions of hyperactivated spermatozoa (Le Lannou et al, 1992). It has been recommended that the CASA instrument used should analyze 50 to 60 images per second for capacitating spermatozoa to maximize the information available about each track (ESHRE Andrology Special Interest Group, 1998).

The proportion of hyperactivated spermatozoa is then determined, either manually by post hoc analysis of specific kinematic values for each spermatozoon, or by the CASA instrument (eg, the SORT function of the Hamilton Thorne IVOS CASA instrument). As stated previously, for human spermatozoa analyzed at 60 images per second, the kinematic definition for hyperactivated motility is VCL $\pm$ 150 μm/s AND LIN $\leq$ 50% AND ALHmax $\pm$ 7.0 μm (Mortimer et al, 1998). For a spermatozoon to be considered hyperactivated, it must meet all of the kinematic requirements of this Boolean argument. The kinematic definitions must be validated for each CASA instrument and for each image sampling frequency used.

There has long been interest in exploring whether a relationship exists between the proportion of spermatozoa that show hyperactivated motility and in vitro fertilization rate, but this has yet to be determined conclusively in a clinical setting. One of the confounding factors in the application of the hyperactivation assessment in a clinical setting was that there is no fixed time after separation of the spermatozoa from seminal plasma when hyperactivated motility occurs because this can differ among men. This has been addressed recently, with the development of a procedure referred to as *HAmax* (Mortimer D et al, 1997). In this test, the prepared spermatozoa were treated with a combination of progesterone and pentoxifylline, which induced essentially all of the spermatozoa that were capable of hyperactivated motility to exhibit this motility at the same time. This means that the test can be run by a schedule, with the motility evaluation step occurring at a fixed time after the sperm preparation step. A preliminary report of the relevance of this test suggested that if very few of the spermatozoa in a sample are induced to hyperactivate in the HAmax procedure, then there is decreased likelihood that the man's spermatozoa will be able

to fertilize his partner's oocytes in a subsequent IVF cycle (Mortimer D et al, 1997). The value of such a test is obvious when the emotional and financial costs of assisted conception treatment are taken into account, although validation of the test remains to be established.

## Conclusions

The benefits of CASA analysis in the clinical laboratory are manifold. With greater understanding of the physiological implications of aspects of sperm motility, it is now possible to predict that a man's spermatozoa may not penetrate his partner's cervical mucus in vivo or that the spermatozoa prepared for an in vitro fertilization attempt will fail to penetrate the vestments of the oocyte, that is, the cumulus oophorus and zona pellucida. The ability to make these predictions is a very important one because it allows for more effective and streamlined clinical management, on the basis of just a few semen assessments. It has been reported that the results obtained for a man's spermatozoa in sperm function tests are still applicable even some weeks later (Sukcharoen et al, 1996), so if these assessments were included in a couple's initial work-up, then the course of treatment would be set from the beginning. This should improve the assisted conception experience for the couple, more effectively direct financial outlay, and coincidentally improve the pregnancy rates for the clinic.

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