Cytometry Part A 71A:308–316 (2007)

A Computerized Spectrophotometric Instrumental System to Determine the "Vertical Velocity" of Sperm Cells: A Novel Concept

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Received 28 August 2006; Accepted 28 December 2006

Background: The presently available cell motility-analyzers measure primarily the "horizontal" velocity and there is no instrument available for "vertical" velocity measurement. This development was based on the turbidimetric method of sperm motility analysis.

Methods: Sperm was layered at the bottom of the cuvette containing buffer solution and exposed to the spectrophotometric light path at different heights to track the vertically moving sperms. The vertical movement was materialized with the development of an electromechanical up–down movement devise for the cuvette accomplished with the help of a cuvette holder-stepper motor-computer assembly. The entire system was controlled by the necessary motion control, data acquisition, and data processing softwares developed for cuvette movement and data analysis.

Results: Using goat sperm as the model a unique computer-based spectrophotometric system has been developed for the first time to determine the average "vertical" velocity of motile cells.

Conclusions: Undertaking upward movement against gravity is much tougher as compared with horizontal movement. Consequently average vertical velocity is expected to be a much better identifying parameter for assessing semen and other motile cell quality. The novel instrumental system developed by us has thus the potential for immense application in human infertility clinics, animal-breeding centres, centres for conservation of endangered species, and also for research work on vertical velocity of spermatozoa and other motile cells, such as bacteria, protozoa, etc.

Key terms: sperm cells; cell motility; vertical velocity; spectrophotometric method; cell motility assay technique; computer-controlled cell motility analyzer

Motility is an important parameter for flagellated or ciliated cells for their survival and propagation. There are different types of unicellular organisms such as bacteria, protozoa etc. that show motility by their swimming property. This motility has been found to be necessary for their virulence and motility of different groups can be differentiated by their respective velocities. Velocity level is also directly related to their infectivity (1–11). In case of spermatozoa velocity is considered as one of the primary determinant factor to predict on the quality and fertilizing ability (12,13). In the present international scenario, microscopic method is the most widely used subjective method for sperm motility analysis. Subsequently, more objective methods were developed, such as light scattering method, laser beam method, multiple exposure photographic method, etc (14–22). But, all the available techniques consider only the "horizontal" velocity and there is not a single instrument available for measuring the "vertical" velocity of spermatozoa. The sophisticated computer aided semen analyzer (CASA) based on microscopic video photographic method is widely used for estimating sperm horizontal velocity from the horizontal plane of a glass slide or haemocytometer or Makler Chamber (23–27). Another objective method of sperm motility analysis is the spectrophotometric/turbidimetric method described by Sokoloski et al. (28) and simplified and made user-friendly by Majumder and Chakrabarti (29). In this method, sperm sample/semen is layered at the bottom of the cuvette filled with adequate buffer to allow the light beam to pass through it, and motility is formulated as the rate of change in optical density/absorbance (at 545 nm) as the sperm

Grant sponsor: Department of Science and Technology, India.
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Published online 23 February 2007 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cyto.a.20382

swim upward into the light path (28,29). It does not rely on arbitrary rating by an individual (30). In the turbidimetric method although the vertically moving cells are considered but it does not give any absolute value of sperm vertical velocity. It is evident from our daily life experience that climbing staircases is much more difficult than walking on horizontal plane. Many such events show that vertical movement is much tougher as it requires more energy since it is a movement against the gravity. It is thus no wonder that much smaller percentage of the spermatozoa show "vertical" motility compared with "horizontal" motility (29). Moreover, in any in vitro fertilization (IVF) program it is a routine practice to select the best sperms by the swim-up technique (31–35) thereby implicating that spermatozoa possessing "vertical" motility have better fertility potential. Therefore, to measure dynamics of motile cells in the vertical plane this novel instrumental system has been developed using caprine (goat) sperm as the model system.

MATERIALS AND METHODS

Modification of Spectrophotometer: Development of Vertical Velocity Measuring System

To measure the vertical velocity of motile cells, the absorbance at different heights of the cuvette with respect to time was required to be acquired. For this purpose the cuvette was to be moved vertically up and down within the spectrophotometric light path. There are different types of spectrophotometers available in the market, in which cuvettes are vertically static, but movable in lateral or circular directions. These lateral or circular movements are incorporated for multiple sampling only but their vertical position remains always fixed. Thus, to make the system adaptable to any spectrophotometer the vertical movement of the cuvette was arranged in two types of spectrophotometers, one with static cuvette holders (DIGISPEC, Model: 110D, SICO India, India), which is laterally movable manually and another with circular moving or rotating cuvette holders (UV-VIS Spectrophotometer, Model: SL-159, ELICO India, Hyderabad, India).

The system assembly in the spectrophotometer with static cuvette holder. In the spectrophotometer with static cuvette holders (laterally movable manually), an electromechanical assembly was developed with a modified cuvette holder coupled with a stepper motor and gear assembly [together forming the cuvette actuator unit (CAU)]. Assembly of modified cuvette-holder with stepper motor for the spectrophotometer with static cuvette holder is shown in Figure 1. The CAU drove the stepper motor and ultimately actuated the electromechanical assembly for upward and downward movements of the cuvette. A cuvette controller unit (CCU) was developed in a personal computer (PC) by installing a control-card. A data acquisition (DAS) card consisting of Analogue to Digital converter was installed in the PC for converting analog data into digital format and acquiring online experimental data. Programmed pulses were generated from the computer, which on arriving at the CCU drove the stepper motor through the stepper motor driver circuit housed in the CAU. This in turn drove the mechanically coupled cuvette holder, which moved the cuvette in the upward and downward directions within the light path, thus exposing it to variable heights (four different heights for this study). Analog output was converted to digital signal and fed to the DAS card, thus the digital data (Absorbance vs. Time data) was stored in the PC. Online data were acquired in the PC from four different heights of the cuvette in accordance with the associated software developed. Block diagram of the entire assembly is shown in Figure 2.

The system assembly in the spectrophotometer with rotating cuvette holder. In the spectrophotometer with circular moving cuvette holders, the technique of exposing the cuvette to the light path in four different heights was accomplished by vertical sliding-movement of the cuvette-load attachment over a circular base of varying heights developed for the purpose. All the six existing cuvette holders from the circular base of the spectrophotometer were removed and a circular and angular disc cam, made of ebonite, with varying heights was fabricated and mounted on the outer surface of the circular base of the cuvette holder. The variation in height was made in such a manner that the cuvette could be exposed to the light path in multiple heights (four for this study). A single cuvette holder was developed, which was attached with a proportionately designed and freely movable metallic load and allowed to roll over a vertically hanging slide. The entire attachment was hanged together at the top of the inner-body of the motor-cuvette holder enclosure, in such a manner that the base of the cuvette-holder can slide up and down over the circular disc cam with varying heights as and when the circular base mounted on the motor rotates. The attached load easily pulled the cuvette-holder down during downward movement. The motion control software (modified with the help of the spectrophotometer manufacturer) controlled the movements and pauses of the circular disc cam with required control signals from the PC. Schematic diagram of the modified cuvette-holder along with modified cuvette-load arrangements for spectrophotometer with rotating cuvette holder is shown in Figure 3.

Development of software for data analysis. Online data was acquired and stored into MS-Access database system in the PC. Once the data are acquired, from the either system (static or rotating), the analytical software performs all the mathematical calculations required for the determination of sperm motility, using a specific mathematical algorithm based on the relation between spectrophotometric absorbance and sperm concentration (described in the result section). Visual Basic, Studio 6.0, Enterprise Edition (VB), was used to develop the analytical software and the user interface. Report generation software was developed with Seagate Crystal 8.0, Developer Edition, which generated the necessary reports on sperm motility as required.

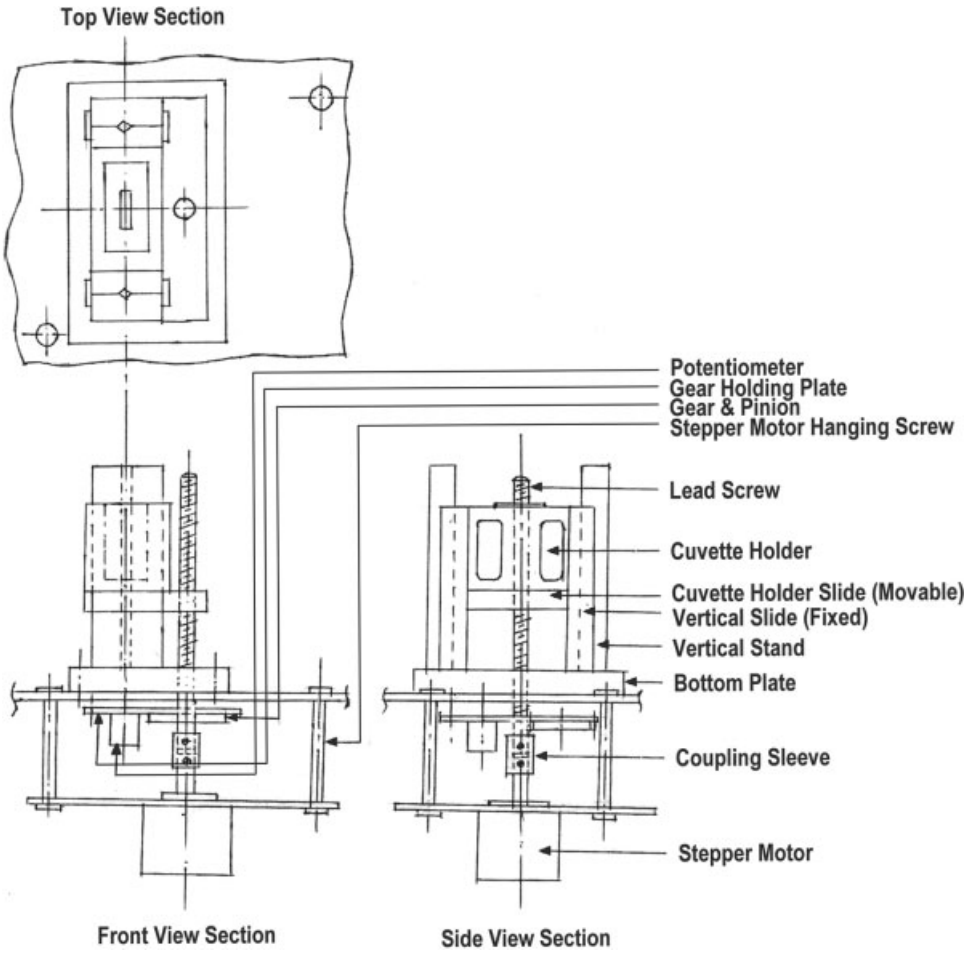

FIG. 1. Schematic diagram of the modified cuvette-holder along with stepper motor for the spectrophotometer with static cuvette holders.

Preparation of Sperm Sample

Goat cauda epididymis was brought from nearby slaughterhouse and sperm was then extracted following standard procedure (29). Cell number was counted microscopically and the concentration was made to be around 200 million cells/ml with modified Ringer's phosphate buffer solution (RPS) free of Ca^{2+} (119 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM glucose, 16.3 mM potassium phosphate buffer, pH 6.9; penicillin, 50 units/ml). 400 μl of this sperm suspension and 100 μl of 10% Ficoll-400 was mixed together so that the total assay volume became 500 μl with a Ficoll-400 concentration of 2% following a procedure standardized in our laboratory. Ficoll (2%) has no adverse effect on sperm motility and it was done so that only the motile cells swims up and not the dead cells (29). This makes the final solution for application in the cuvette.

Spectrophotometric Assay Procedure for Sperm Vertical Velocity

At the beginning, the initial conditions of the spectrophotometer were adjusted. The wavelength of the spectrophotometer was set at 545 nm (produces highest peak for sperm sample). The total scan time was given within a range of about 3–20 min to obtain a saturation curve for absorbance vs. time. The time interval between the start of each set of scan was set at 1 min (60 s) so that every individual scan (1st, 2nd, 3rd, or 4th) in subsequent cycles started exactly at an interval of 60 s. There was no delay in time as the movements are all well synchronized. Approximately 0.9 s is required to move the cuvette from one height to the adjacent one and the entire up and down movement to complete one cycle of scanning takes about 6 s but it does not cause any interference in the scanning intervals. The cuvette was then filled with 1.5 ml (1,500 μl) of the modified RPS and placed in the cuvette holder of the spectrophotometer. This was important because the light beam in normal condition must pass through the uppermost part of the solution. The computer interfacing software was initiated at this stage when the spectrophotometer recorded the reference data and paused for adding sperm sample. After this with the help of a 500 μl Hamilton Syringe 50 μl of the prepared sample from the test tube was layered slowly and delicately at the bottom of the cuvette. The shutter of the spectrophotometer was closed as quickly as possible and the associated computer interfacing software of the instrument

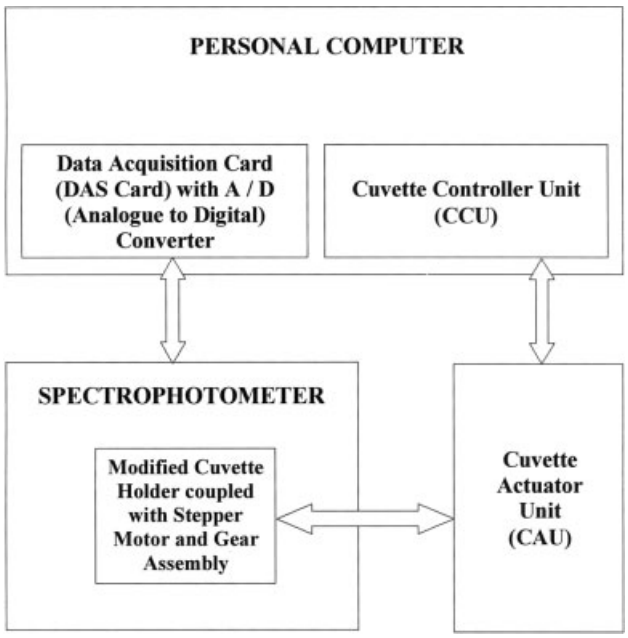

FIG. 2. Block diagram of the entire assembly for the spectrophotometer with static cuvette holders.

was initiated. The experimental data, absorbance vs. time, was acquired at four different heights of the cuvette during each cycle of time scanning.

RESULTS

Achievement from Cuvette Holder Modification

Modifications in the cuvette holder arrangement allowed the cuvette to be exposed at four different heights, each 2 mm apart from the other, starting from the base of the cuvette (Fig. 4). Data was collected from all the four heights at the end of every minute upto a user defined time span. Thus, light beam passing through those four heights almost at the same time recorded the change in the concentration of sperm population at those heights with respect to time. A literally pictorial view of the vertically moving cells in the cuvette with the passage of time could be ascertained using the acquired database i.e., cell concentration at different intervals of time and at different heights could easily be obtained (Fig. 5).

Calculation of Vertical Velocity

As the positions of different batches of sperm cells (in terms of absorbance) at different time intervals were obtained, so, a group of sperm cells can be traced for its upward movement throughout its upward journey. Thus, the time required by a particular batch to complete its journey can be easily determined from the absorbance vs. time plot (Fig. 5). Then vertical velocity of different groups of sperm cells could easily be calculated just by using the simple formula:

Vertical Velocity = Vertical distance traversed by a particular group of sperm/Time elapsed.

All the heights or distances are known (constant factor), so, the time (variable factor) required by a group of cells to travel from base of the cuvette to different heights and in between different heights were calculated to determine the vertical velocities and average was taken in both the modes. Average of two average vertical velocities for a particular group of cells (selected preferably from initial stages for accuracy) gave its average vertical velocity for the entire journey.

As for example, from Figure 5B it can be seen that at 120 s the absorbance is 0.033 at the topmost height (H0/8 mm). Then, from the plot it was found that the same absorbance existed or crossed heights H3 (2 mm), H2 (4 mm), H1 (6 mm) at 18, 24, and 44 s, respectively. So, the Table 1 shows the method of average vertical velocity calculation.

Vertical Velocity vs. Horizontal Velocity

The vertical and horizontal velocities were determined side by side in the new instrumental system and CASA (Version: 10, HTM-IVOS CASA System, Hamilton Thorne Research, Beverly, MA) with the same samples. The

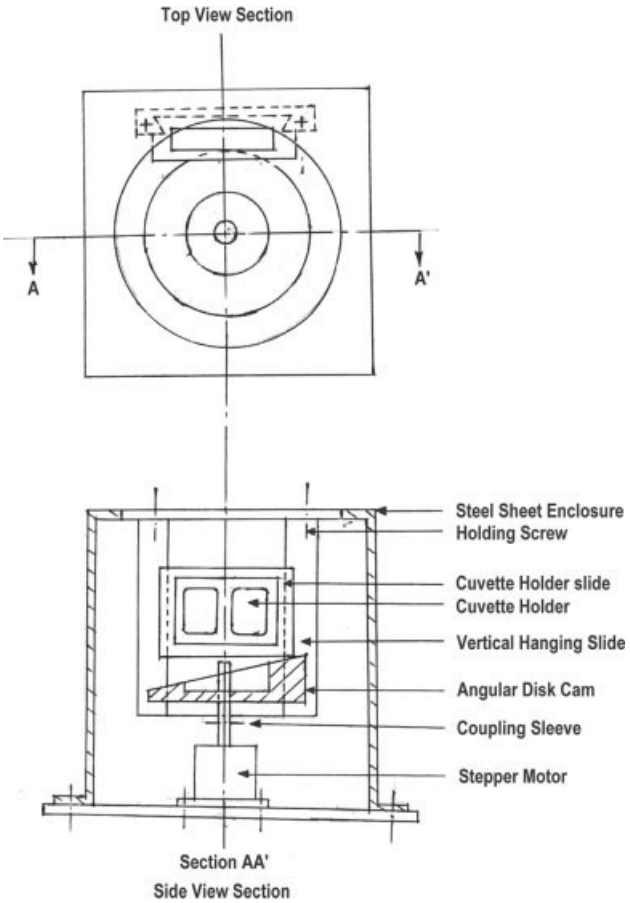

FIG. 3. Schematic diagram of the modified cuvette-holder along with modified cuvette-load arrangements for the spectrophotometer with circular moving cuvette holders.

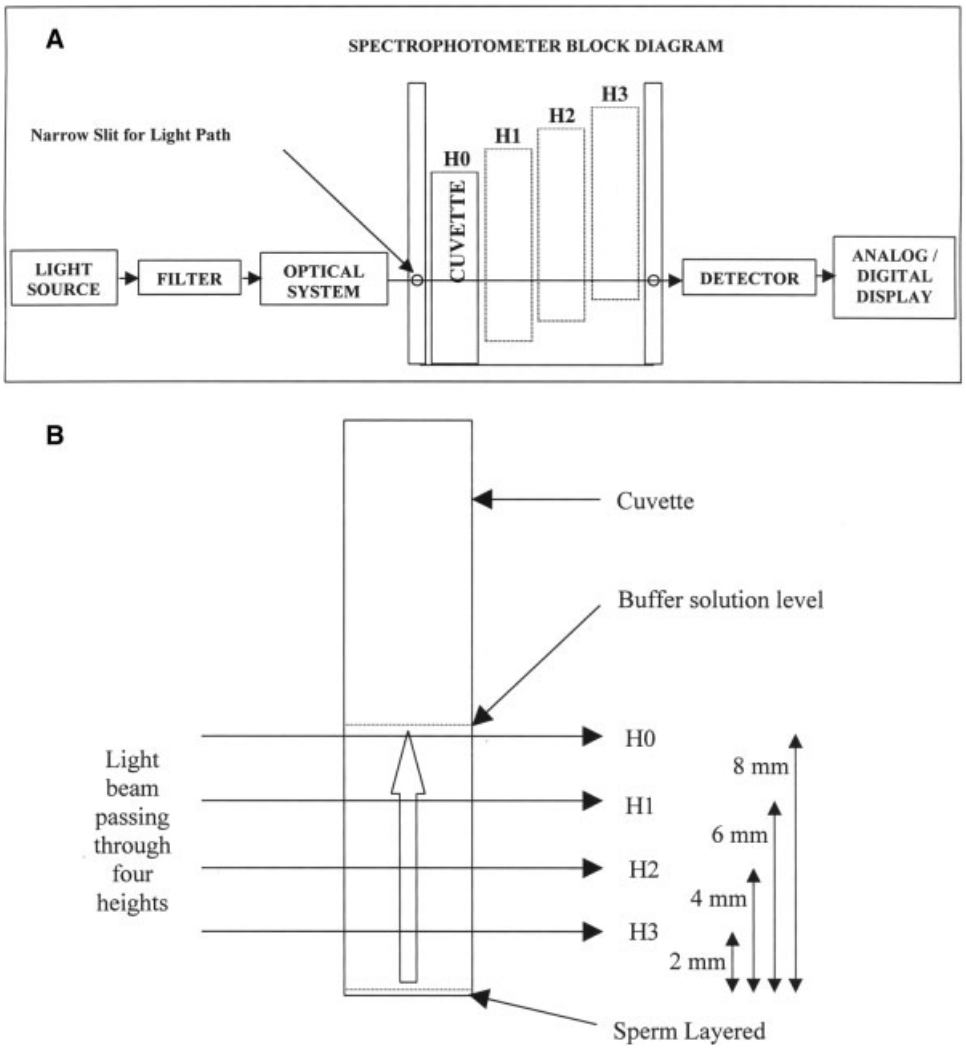

FIG. 4. A: Schematic block diagram showing the spectrophotometric system with the positions of the cuvette on vertical movement. B: Schematic diagram of the cuvette describing the buffer solution level, position of the sperm layer in the cuvette and the heights at which the cuvette is exposed to the spectrophotometric light beam.

average vertical velocity (VAV) obtained from the new system is shown side by side with the average path velocity (VAP) obtained from the CASA in Figure 6. The average of vertical velocity was considered as sperms can come up from the base of the cuvette following any random paths that may be straight, angular, curved, swirling, zigzag, etc. with accelerations and decelerations. The VAP of CASA was found most suitable to display side by side as it also gives the average velocity of the sperm from the net displacement due to movement in different directions. The trend of this result is such that the value of the average vertical velocity was observed to be lesser than the corresponding average horizontal velocity. The difference between the corresponding horizontal and vertical velocity values were found to be varying from sample to sample as shown in Figure 6. The horizontal velocity ranged between 60 and 160 μm/s (approx.), whereas the vertical velocity ranged between 50 and 100 μm/s (approx.). It has already been reported earlier that the spectrophotometric values did not necessarily correlate with microscope assessments of forward motility, as they are parameters of two totally different dimensions (29,30).

As shown earlier (29), vigorously forward motile cells show high order of motility values when analyzed by microscopic as well as spectrophotometric methods. Highly motile sperm samples were stored at room temperature [(32 ± 1)°C] for varying periods (7 h) prior to assay of motility by both the microscopic and spectrophotometric methods (Fig. 7). The freshly prepared sperm samples as expected showed high order of motility by both the methods. However, on weakening of motility because of the storage, the relative loss of sperm motility was much sharper when analyzed by the spectrophotometric method than by the microscopic method. It is striking to note that even after 7 h of storage as much as 20% (approx.) cells showed weak forward motility by the microscopic method though these cells did not manifest any detectable vertical movement analyzed by the spectrophotometric method (Fig. 7). The results clearly demonstrate that like the human beings, microscopic cells also

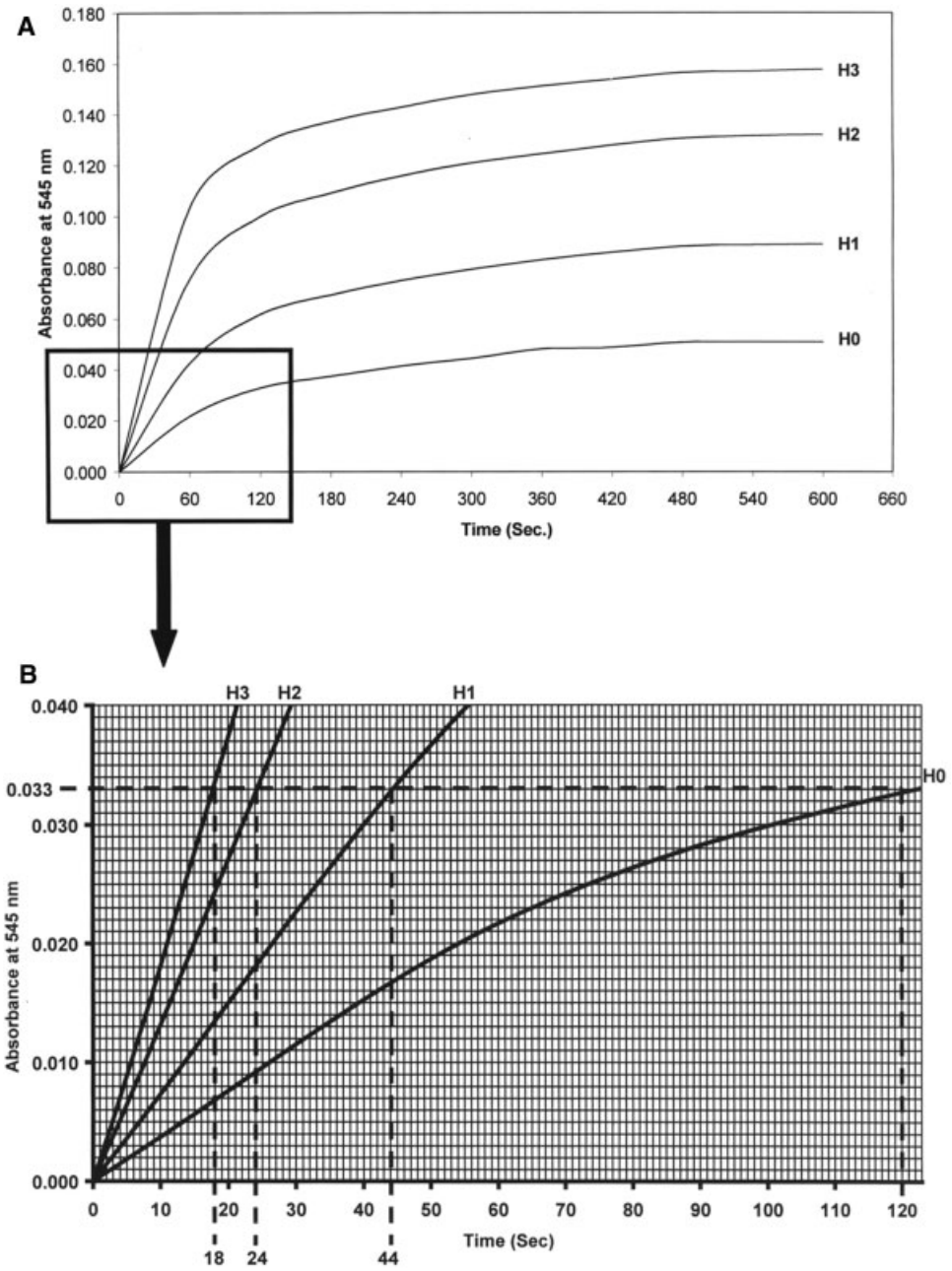

FIG. 5. A: The entire Absorbance Vs time plot showing the curves at different heights. B: Highlighted part of Fig. 5A, as shown in the rectangular box, has been magnified to calculate the time required by the sperm cells to reach specified heights of the cuvette.

face lots of difficulties to perform vertical movement. Similarly, results were also obtained when the sperm motility was weakened with *p*-chloromercuribenzoic acid: a sperm motility inhibitor (29) (data not shown).

DISCUSSION

It is well documented that sperm motility is essential for the natural fertilization process. The age-old method of sperm motility measurement is the microscopic method that essentially measures horizontal motion of the cells and this method gives only a subjective assessment of sperm motility. Subsequently several objective methods have been developed to measure sperm horizontal movement, the CASA being the most widely used one (14–27). Although CASA gives multiple sperm motility parameters, their correlation with fertility potential of spermatozoa is not well defined (23–27). Some workers have analyzed sperm cells showing upward movement against the gravity by spectrophotometric methods (28,29). The major limitation of these methods is that they are not computer-based (i.e. manual) and that they provide only arbitrary units of sperm movement. Consequently the dream of reproductive biologists to determine sperm vertical velocity in absolute terms remained unfulfilled. This study reports for the first time a novel computer-assisted method to fulfill the long cherished desires of the scientists. This

Table 1
Method of Average Vertical Velocity Calculation

Travel course (from & to)	Distance (μm)	Traveling time (Sec)	Vertical velocity (μm/sec)	Average (μm/sec)	Average vertical velocity (μm/sec)
Base to different heights					
Base to H0	8000	120[a]	67		
Base to H1	6000	44	136	89	
Base to H2	4000	24	42		116
Base to H3	2000	18	111		
In between different heights					
Base to H3	2000	18	111		
H3 to H2	2000	6	333	143	
H2 to H1	2000	20	100		
H1 to H0	2000	76	26		
Base to different heights					
Base to H0	8000	180[b]	44		
Base to H1	6000	51	118	103	
Base to H2	4000	27	148		112
Base to H3	2000	20	100		
In between different heights					
Base to H3	2000	20	100		
H3 to H2	2000	7	286	121	
H2 to H1	2000	24	83		
H1 to H0	2000	129	16		

[a]At 120 sec the absorbance is 0.033 at the topmost height (H0 or 8000 μm) (Fig. 5B).
[b]Similarly at 180 sec the absorbance is 0.037 at the topmost height (H0).

has been accomplished by designing an electromechanical system comprising a modified cuvette holder and a stepper motor (Figs. 1 and 3) tailor-made to fit inside the small area of the standard spectrophotometer. Introduction of this electromechanical system (Fig. 2) permitted us to analyze vertically-moving sperm cells at different heights because of the controlled upward/downward movement. Another important component of this innovation are three custom designed softwares developed for cuvette movement, data acquisition at different heights of the cuvette and data analysis purposes. The mathematical model developed for average vertical velocity calculation is also very simple (Table 1). Moreover, all the motility analyzers till date, including CASA, never considered the importance of motility in vertical direction and no attention was given to the possible differences from the horizontal motility. This is for the first time that movement of cells in the vertical plane has been considered for analysis and an instrumental system has been developed for automated determination of average vertical velocity of motile cells.

Healthy motile cells having more velocity are expected to be more active or functional. In case of sperm it has to travel the entire female reproductive tract to fertilize the ova. The entire journey has several obstacles and hostile conditions, such as the viscous cervical mucus and uterine

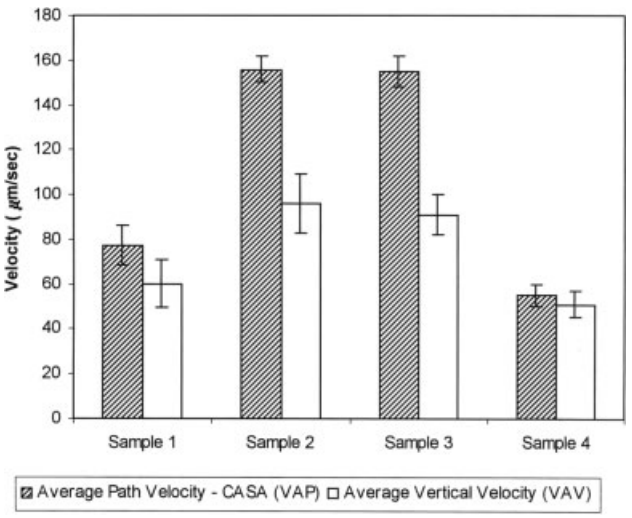

FIG. 6. Corresponding sperm velocity results of the same samples from CASA (Average Path Velocity) and New System (Average Vertical Velocity) displayed side by side. Result showing Mean ± SEM of three experiments.

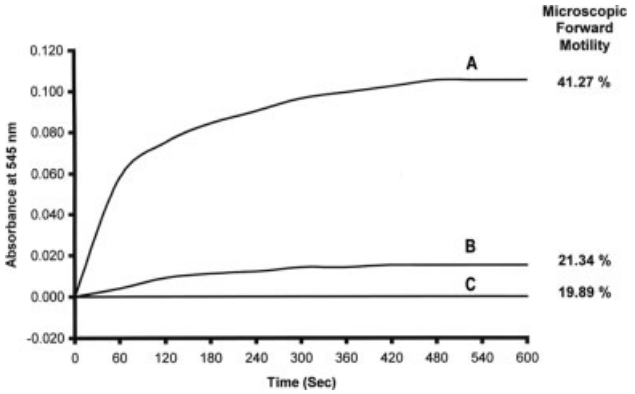

FIG. 7. Corresponding spectrophotometric and microscopic studies of sperm cells weakened by keeping in room temperature for varying periods (7 h) in microscopic and spectrophotometric studies. A: 0 h, B: 6 h, C: 7 h.

fluid, the acidic pH, etc., which prevents the easy movement of sperm (36–38). Part of this journey may also be in the vertical direction (e.g. human), which requires a movement against the gravity. Therefore, only the healthiest cells are expected to overcome all the hurdles and fertilize the ova. Thus, it is likely that average vertical velocity, in comparison with horizontal velocity, is expected to be a better identifying parameter for gradation of semen samples according to quality. The vertical motility of sperm cell is important as we find that in any IVF program swim-up technique is used to select the healthiest spermatozoa for ensuring more success in fertilizing the ova (31–35). It is also evident from our results (Fig. 7) that weak sperm cells possessing significant horizontal movement (microscopic assay) fail to register any appreciable vertical movement (spectrophotometric assay). This is because cells cannot take up vertical motility against the gravity due to weak health. VAV of same sample has been found to be comparatively lesser than its corresponding average path (horizontal) velocity (VAP) measured by CASA (Fig. 6). This also supports the importance of vertical velocity as an index to measure the health of a motile cell.

As mentioned before all the motility parameters given by the horizontal motility-assessing instruments including CASA are not yet well correlated with the fertilizing efficacy of the male gametes. The novel instrumental system developed by us can be used more effectively for semen quality evaluation. Correct assessment of semen quality will be extremely helpful for better treatment of human infertility and planning of animal breeding programmes. For example, a single ejaculate of top quality semen (based on "average vertical velocity") will result in more successful artificial inseminations by selecting minimal dose of sperm without causing any wastage. So, it will be very much useful to various human infertility clinics, animal breeding centers, research laboratories and centers for conservation of endangered species, etc. This study will open a new avenue of research regarding molecular basis of cell movement with special reference to dynamics of motile cells in the vertical plane. Measuring vertical velocity or vertical vector of a cell is a novel idea that may be extended to a variety of other motile cells (e.g., protozoa, bacteria, etc.) and particles. It is expected that, once this instrumental system is marketed and gets exposure, various other fields of its usage will be revealed. To acquire the intellectual property right, applications have already been filed for national and international patents (39,40).

ACKNOWLEDGMENTS

The authors express their sincere thanks to Dr. Archana Bhardwaj Shiva and Dr. S. Shivaji of Centre for Cellular and Molecular Biology, Hyderabad, India, for their cooperation in performing the experiments with CASA. They also wish to express their gratitude to The Directors of Indian Institute of Chemical Biology and Indian Association for the Cultivation of Science, Kolkata, India, for taking interest and encouragement for successful completion of this scientific project work.

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