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# Objective evaluation of ram and buck sperm motility by using novel sperm tracker software

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Abstract

This work offers researchers the first version of an open-source sperm tracker software (Sperm Motility Tracker V1.0) containing a novel suit of algorithms to analyze sperm motility using ram and buck sperm as models. The computer-assisted semen analysis (CASA) is used in several publications with increasing trend worldwide in the last years, showing the importance of objective methodologies to evaluate semen quality. However, commercial systems are costly and versatility is constrained. In the proposed method, segmentation is applied and the tracking stage is performed by using individual Kalman filters and a simplified occlusion handling method. The tracking performance in terms of precision (number of true tracks), the percentage of fragmented paths and percentage of correctly detected particles were manually validated by three experts and compared with the performance of a commercial motility analyzer (Microptic's SCA®). The precision obtained with our Sperm Motility Tracker was higher than the one obtained with a commercial software at the current acquisition frame rate of 25 fps ($p<0.0001$), concomitantly with a similar percentage of fragmentized tracks ($p=0.0709$) at sperm concentrations ranging 25 and $37 \times 10^6$ cells/ml. Moreover, our tracker was able to detect trajectories that were unseen by SCA®. Kinetic values obtained by using both methods were contrasted. The higher values found were explained based on the better performance of our sperm tracker to report speed parameters for very fast motile sperm. To standardize results, acquisition conditions are suggested. This open-source sperm tracker software has a good plasticity allowing researchers to upgrade according requirements and to apply the tool for sperm from a variety of species.

## Introduction

Motion analysis on quality assessment of semen samples is of great importance for the positive association with male fertility and because it is in one of the most affected parameter after cryopreservation. However, sperm tracking is quite complex due to cell collision, occlusion and missed detection. Computer-assisted semen analysis (CASA) systems are used in several publications with an increasing trend worldwide in the last years, showing the importance of objective methodologies to evaluate semen quality and predict fertility. It is well-known that CASA systems are commonly used for determination of sperm quality from various species (Billard & Cosson 1992, Dietrich *et al.* 2005), cryopreservation effectiveness (Cueto *et al.* 2016), toxicity bioassays, prediction of fertility potential or research related to basic sperm biology (Muiño Otero 2008, Muiño 2008, Buzón Cuevas 2014).

CASA systems provide sequential digital images of each spermatozoa track allowing individual motion analyzing thus facilitating a rapid, precise and accurate assessment of several and meaningful kinetic measurements (Verstegen *et al.* 2002, Amann & Waberski 2014) that are considered as objective and reproducible, while using identical instrument settings. On the other hand, it has been recognized that among commercial software disadvantages, one can mainly list high cost, need to regular upgrade and dramatic changes influenced by different settings that are not well documented in publications (Schleh & Leoni 2013). Even when each lab standardizes its own conditions, the setup of the parameters is crucial to allow comparisons between different studies and to obtain reproducibility as well as consistency of internal and external controls (Holt *et al.* 1994, Fraser 1998). Since there are many factors affecting CASA performance (Broekhuijse *et al.* 2011), the methodologies and system specificities (equipment, chamber, plate temperature and acquisitions details) have to be fully and clearly described (Verstegen *et al.* 2002). However, these details are not often given in most publications. Moreover, the accuracy of CASA results is intrinsically dependent of the range of sperm concentrations analyzed (Muiño Otero 2008, Muiño 2008, Talarczyk-Desole *et al.* 2017).

Another fact that has to be considered is that motility estimates and concentration using CASA systems are highly influenced by the counting chamber (Hoogewijs *et al.* 2012, Palacín *et al.* 2013). Besides spermatozoa speeds vary according to each species, the choice of a particular acquisition velocity is under discussion, since the selected frame rate affects the measure of several kinetic parameters (Davis & Katz 1992, Verstegen *et al.* 2002). Verstegen *et al.* (2002) described that trajectories are not well detected when setting of maximum velocity is too low, in these cases the software generates wrong trajectories since it connects points belonging from different spermatozoa tracks. In most of the cases, a good measure of a high curvilinear velocity value is due to a good frame rate setting.

Concerning costs, there are also some open source systems that are widely useful in sperm motility analysis, e.g. National Institutes of Health has developed a CASA plugin for the ImageJ software (Wilson-Leedy & Ingermann 2011) that has been especially adapted for the kinetic analysis of fish sperm (Verstegen *et al.* 2002) but also validated for mammalian sperm

(Giaretta *et al.* 2017). Disadvantages of this method include many manual settings, needing to apply different thresholds to each video.

The aim of this work was to develop an automated particle detection tool and a suite of tracking algorithms to analyze motility parameter characteristics using ram and buck sperm as models. Our tool has the clear advantage that is plausible to be extrapolated to other species due to its plasticity to perform changes depending on the researcher's objectives and the intrinsic characteristics of the samples. Moreover, this prototype is useful to track each spermatozoon since the corresponding trajectory is drawn step by step through the images sequence.

In this way, we developed a sperm tracker software containing a suite of algorithms for sperm motility analysis that includes the stages of detection (frame to frame), tracking and motility analysis for videos of ram and buck sperm cells. A manual validation was performed to compare the tracking performance of our algorithm with that of an available version of the Microptic's Sperm Class Analyzer-SCA® over the same videos. This work offers an open-source software to evaluate semen motility for researchers in the reproductive field

## MATERIALS AND METHODS

### Samples collection

Animal handling was performed in accordance with Spanish Animal Protection Regulation, RD 53/2013, which conforms to European Union Regulation 2010/63. Blanca Celtibérica buck and Manchega ram (age > 1.5 years) were maintained in a semi-free ranging regime at El Campillo (Elche de la Sierra, Albacete, Spain) or at experimental farm of University of Castilla-La Mancha, respectively. The collection of ejaculates was performed using two different methods: artificial vagina for ram (5 males) or electroejaculation for buck (5 males), according to the guidelines RD 841/2011 and protocols previously described (Marco-Jimenez *et al.* 2008, Jimenez-Rabadan *et al.* 2012). Ram samples were collected and pooled whereas samples from buck were analyzed individually.

Sperm concentration was calculated by Bürker chamber counting and adjusted to 30 x $10^6$ spermatozoa/ml for ram and 20 x $10^6$ spermatozoa/ml for buck with a phosphate buffer (PBS) at 37 °C.

Experimental procedure

Objective motility was assessed with a Makler® counting chamber (10 µm depth) and samples were observed using a 10 X objective (negative phase contrast field). Each analysis captured several fields with a Basler A302fs digital camera (Basler Vision Technologies, Ahrensburg, Germany) connected to a computer by an IEEE 1394 interface. The image size was 768 x 576 pixels. The acquisition frame rate was set in 25 frames per second (fps) videos which were simultaneously analyzed by Computer Assisted Semen Analysis (CASA) using the Sperm Class Analyzer software (SCA® 2002, Microptic, Barcelona,Spain) and by our sperm motility tracker software. Buck sperm tracking videos produced by our algorithm are available at Vimeo homesite (see references https://vimeo.com) The motility parameters assessed are described in section D (Motility Parameters and Motility analysis).

Algorithm development

A. Detection of the Cells Head

Image processing algorithms were developed in C++ with the Netbeans IDE and using the OpenCV 3.2.0 library. A detection method similar to the one have been used by Buchelly *et al.* (2016) for cells segmentation was used but with a highlighting step due to opening Top-Hat (Serra 1982). For the Top-Hat transform we used a circular structuring element with the sufficient size to enclose one spermatozoon head (11x11 pixels). The fixed threshold to obtain the binary image was set to 30. The structuring element used for the binary morphological filter is circle shaped and it has a size of 5 x 5 pixels to remove little noise points, sharp features like the sperm tails, and to separate some particles.

B. Concentration measurement

Cells concentration was determined for each sample video as the average number of cells in each frame per square millimeter (cells/cm3), according to (1):

$$D = \left(10^8 \frac{\mu m}{cm^3} \frac{L^2}{d}\right)\left(\frac{\sum_{k=1}^{N} n_k}{w.h.N}\right) \qquad (1)$$

Here, *L* and *d* are the setting parameters and depend on the experimental conditions: *L* is the length of the side of the gride square in pixels and *d* is the counting chamber depth in micrometers, *w* and *h* are the image width and height respectively in pixels. So, the first factor in (1) refers to the transformation of the lengths from pixels to metric units. By the other hand, the second factor shows the average number of cells in the video sequence determined by the numbers of cells in each frame ($n_k$) and the total number of frames, *N*.

C. Sperm cells tracking

In order to define an object's model, kinematic variables, shape or geometric descriptors, contours, gray levels or textures can be considered (Lucena López 2003, Azari *et al.* 2011, Liu *et al.* 2013, Jeong *et al.* 2014, Sahbani & Adiprawita 2016). From this set of data, the model is represented by the state Xi of the system at the instant i with a given number of degrees of freedom (Lucena López 2003). Our object's model consisted only on the head centroid or mass center coordinates (Gárate Polar 2015) and its velocity components. As it doesn't rely on the geometry of the cell head or on gray levels information, a spermatozoon was treated as a point particle. The dynamics of the system was studied with a first order model, i.e. positions and velocities are measured to predict the future positions. The trajectory of the j-the particle was defined as the discrete collection of positions at all the instants i. The velocity vector of a particle j between the instants i-1 and i, was determined using (2):

$$V_{i,j} = \left(\mathrm{x_{i,j}} - \mathrm{x_{i-1,j}}\,,\ \mathrm{y_{i,j}} - \mathrm{y_{i-1,j}}\right) = (\mathrm{u_{i,j}}, \mathrm{v_{i,j}}) \quad (2)$$

The model of the dynamics offers an a priori distribution of probabilities about all the possible configurations of the current state of the system p(Xi) having into account the estimated distributions for the previous instants p(Xi-1) to P(Xi-n). By the other hand, the temporal fusion method uses the Bayesian framework to integrate the a priori probabilities with the set of measures Z (coordinates of the centroids of the detected cells in the current frame) to find the a posteriori distribution (Lucena López 2003) given by (3):

$$p(X_i|Z)\ \alpha\ p(Z|X_i)\,.\,p(X_i) \quad (3)$$

The objective was to maximize p(Xi|Z) in order to estimate the new state (Lucena *et al.* 2010) or to give the correct labels to the new detected particles according to the previous known ones. In (3), the value p(Z|Xi) is the observation model. Despite of its limitations, Kalman filter is ideal to use with Gaussian and unimodal distributions (Lucena López 2003), assuming constant or low acceleration rates (Vinaykumar & Jatoth 2014). We associated Kalman Filter to each detected particle to predict its future position (Catlin 1989, Azari *et al.* 2011, Jeong *et al.* 2014), as follows: let the state of a single particle j at the instant i, and the measurement vector. The state and the measurement are estimated by using (4) and (5):

$$\mathrm{X_{i,j}} = \mathrm{A}_j X_{i-1,j} + \varepsilon_j \quad (4)$$

$$\mathrm{Z_{i,j}} = \mathrm{H}_j X_{i-1,j} + \delta_j \quad (5)$$

Where Aj is the transition matrix for the particle j and has the values shown in (6). By the other hand, Hj is the measurement matrix and for this work it corresponds to the identity matrix I ∈ $R^{4x4}$; $\varepsilon_j$ and $\delta_j$; and are vectors corresponding to the process noise and the measurement noise respectively. The noise vectors are initialized with a constant value and updated during the execution time.

$$A_j = \begin{pmatrix} 1 & 0 & 1 & 0 \\ 0 & 1 & 0 & 1 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \end{pmatrix} \quad (6)$$

We used the built-in functions included in the OpenCV library to create and use Kalman predictors. Each of them functions in a cyclic process that consists in three stages and each stage is complemented with a particular routine for our own purposes. First, the system obtains real measures of the state variables and compares them with the measures predicted by the Kalman filters in the previous iteration to do the association by a minimal distance criterion and thus get indirectly the maximization of the a posteriori distribution $p(Xi,j,Zj)$ for each particle. The result of the first stage gives the cell path over which the motility analysis described in the following section is performed. In the second stage, the system gives to the Kalman predictors the new real data to correct the state $Xi,j$ and to update the error vectors $\varepsilon j$ and $\delta j$ and the covariance matrices that are involved in the inner operations. At the third stage, Kalman filters predict the possible future location of each labeled particle by having its state in the current instant, i.e. finding the values of $Zj$ that maximize $p(Zj|Xi,j)$ and that is used in the first stage of the next iteration

D. Motility Parameters and Motility Analysis

Motility of each spermatozoon was defined by its current head velocity descriptors (Muiño Otero 2008, Muiño 2008, Buzón Cuevas 2014):

- Curvilinear Velocity (VCL): Velocity over the total distance moved in the path length, i.e., including all oscillations that occur in the head track. A ram spermatozoon is considered immotile if it has a curvilinear velocity less to 10 µm/s, according to (7).
- Average Path Velocity (VAP): Velocity over a calculated, smoothed (low pass filtered) path, i.e., a shorter distance than that used for calculating VCL.
- Straight-Line Velocity (VSL): Velocity calculated using the straight-line (Euclidean) distance between the beginning and end of the sperm track.
- Amplitude of Lateral Head Displacement (ALH): The average value of amplitude of the oscillatory movement of the sperm head in each beat cycle.

- Beat Cross Frequency (BCF)-The frequency with which the actual track crosses the smoothed track (regardless of the oscillation direction).
- Straightness (STR, %): Measure of the oscillation of the curvilinear path with respect to the average trajectory, calculated as VSL /VCL × 100. Indicates the straightness of the middle path.
- Linearity (LIN, %): Relationship between the straight-line velocity and the curvilinear velocity expressed as VSL/VCL x 100)
- Oscillation (WOB, %): It is a measure of the oscillation of the curvilinear trajectory with respect to the average trajectory, calculated as VAP / VCL × 100).
- Total motility (%): percentage of sperm having a curvilinear velocity (VCL)> 10 µm / s.
- Progressive motility (MP, %): percentage of sperm presenting movement with a straightness index (STR) ≥ 80% within the sample.
    - Statics: VCL <10 µm/s.
    - Low progressive: 10 < VCL <45 µm/s.
    - Mid progressive: 45 < VCL <75 µm/s.
    - Rapid: VCL > 75 µm/s.

As described by other state of the art works (Rojas *et al.* 2012, Liu *et al.* 2013, Gárate Polar 2015, Hidayatullah *et al.* 2015), the discrete set of positions for each spermatozoon head (j-th particle), VCLj was obtained as the mean curvilinear velocity, as described by (7), using the notation defined in the previous sections:

$$VCL_j = \frac{(8.1\mu m).Fr}{L\,(n_j-1)} \sum_{i=1}^{n_j-1} \sqrt{u_{i,j}^2 + v_{i,j}^2} \qquad (7)$$

where Fr refers to the frame rate in frames per second, L is the side length of the grid square given in pixels used in (1), nj is the number of points of the j-th particle path, ui,j and vi,j are the components of the velocity defined in (2), for the nj-1 intervals.

VAP calculation depends on the particular method used for obtaining the smoothed path. Our proposed system uses the method mentioned in Hidayatullah et al. (2015) for smoothing the path and accordingly ALH and BCF parameters.

Manual validation

The variables considered were the number of total trajectories, the precision defined as the number of correct paths over total paths detected (8) and the percentage of fragmented trajectories.

$$\text{Precision} = \frac{TP}{TP + FP} \quad (8)$$

Where TP represents the number of true positives or good tracks and FP is the number of false positives or wrongly assigned tracks. To classify a track as good or bad we used the criterion of three independent expert biologists that performed the manual validation for each path considering whether the labels were correctly conserved during occlusion states.
The percentage of correctly detected particles is defined as the number of detected sperm over the total particles labeled by each software according to the criteria of three experts.

Statistical analysis

Data were analyzed by GLMM (generalized linear mixed effect model) to determine statistical significance between both software (Zuur *et al.* 2009). Data associated to cell percentages were analyzed through models with binomial distribution, whereas the number of trajectories were analyzed by models with Poisson distribution. Velocities were analyzed with Gaussian error distribution. Normality of residuals was assessed by plotting theoretical quantiles versus standardized residuals (Q–Q plots). Homogeneity of variance was evaluated by plotting residuals versus fitted values. All analyses were performed using R software version 3.3.3

(RCoreTeam 2017), with the “lme4” package for Poisson and binomial models and the “nlme” package for Gaussian models (Bates *et al.* 2015, Pinheiro *et al.* 2017). For all analyses, statistical significant differences were determined at $p<0.05$.

Sperm motility tracker software V 1.0

The software is free and an executable version will be provided upon request (acesari@mdp.edu.ar). The software’s user interface provides a step-by-step guide for users. Important instrumental considerations and settings for users are also included (Table 1). Running times are suitable for standard laptop computers with i3 processor and at least 3 GB of memory. Screen resolution can vary between 1280x800 and 1920x1080.

The input to our algorithm software is a sequence of time-lapse images currently encoded either as an MP4, AVI or MOV video file of 5 s acquired at 25 fps. The output of the algorithm is a database (.XLSX) containing the set velocity parameters, population parameters and sperm concentration; a movie (.AVI) with the complete tracks and an image (.BMP) of the tracks.

Results

Particles firstly detected and localized with the highest possible accuracy were linked to form particle trajectories. Detected particles had a near elliptical shape although their areas had a low number of pixels (Fig. 1). The small size let us to approximate the spermatozoa heads as point particles and not to consider their shapes as shown within the region in which we could observe centroids detection (Figure 2). As shown, the intensity degradations avoided the complete detection of the particles’ shape and thus, this supported the idea of working with the point particle model. It was possible to measure sperm concentration by using (1), having a range of particle concentrations between12.64 x $10^6$ cells/ml (38.83 cells/frame) and 42.29 x $10^6$ cells/ml (129.92 cells/frame) for the considered samples (Table 2) consistent with sample adjustment (Material and Methods, Sample collection).

In order to evaluate the tracking performance, the trajectories detected by our proposed algorithm were compared to the ones found by Microptic's – SCA® for two kind of sperm samples: buck and ram fresh ejaculates. A high percentage of the paths tracked by SCA were also followed by our algorithm, and moreover the number of cells followed by the sperm tracker software was higher than the one obtained with the SCA® motility software for both kind of samples (Table 2), suggesting that several sperm particles were only tracked by our method (Fig. 3). The percentage of tracked particles that do not correspond to spermatozoa can vary depending on the quality of the sample, on how clean is the media or on the image quality. In this case, the percentage of correctly detected particles of our proposed method was even higher than the percentage for SCA (Table 2, $\chi 2= 489.61$, Df= 1, $p<0.0001$ for ram and $\chi 2= 6.19$, Df= 1, $p=0.0128$ for buck). Even it is indeed an error, it must be considered as possible and for this reason provided that the percentage of undesirable particles is low, both software are equipped with a tool allowing manually curation or elimination of these labels. Regarding the number of evaluated cells, i.e. those automatically detected and also visually tracked by each expert; the proposed method was higher than the SCA® module for both species ($\chi 2= 450.75$, Df= 1, $p<0.0001$ for ram samples and $\chi^2= 56.66$, Df= 1, $p<0.0001$ for buck samples analysis, Table 2).

There are in the literature some measures that allow the performance evaluation for tracking systems, but the ideal disparity test should be given by the comparison with a ground truth or the point to point comparison of each instant for all the tracks, as done by Fang *et al.* (2017) , Philip *et al.* (2014) or Chau *et al.* (2004). Unfortunately, the SCA® module does not offer that information to compare the differences between each path with the one obtained by our proposed method. According to the criterion of the experts, trajectories were classified as good or bad considering whether the labels were correctly conserved during occlusion states. In this way, our system allows to identify and draw each sperm trajectory frame to frame, representing an advantage over other commercial systems (see Sperm tracking videos). In terms of performance, a 5 s video showing 120 cells/field acquired at 25 fps is enough to produce a complete data sheet by this algorithm in 30 ms using a 1.3 GHz Intel Core i3 processor with 3 GB 1600 MHz DDR3 RAM. Precision and percentages of fragmented paths were evaluated to compare each system through a manual tracking by three independent experts (Table 2, Fig. 4). We showed that the performance of our method is similar to the one measured for the Microptic's SCA® Motility module, with a better occlusion handling evidenced by the higher precision (χ2= 151.03, Df= 1, p<0.0001, Fig. 4A) and a similar percentage of fragmented ram sperm tracks (χ2= 3.26, Df= 1, p=0.0709, Fig. 4C). On the contrary, for buck sperm samples, Microptic's SCA® Motility module showed better precision and lower fragmented tracks that our method (χ2= 16.99, Df= 1, p<0.0001 and χ2= 95.95, Df= 1, p<0.0001 respectively, Fig. 4B and E). When the precision of each system or the percentage of fragmented trajectories is plotted depending on the particles concentration, the better precision of our algorithm can be observed at higher concentrations, while SCA® was more successful for low concentrations ranges (Fig. 4C and F).

The dataset of kinetic values obtained by using both methods over the same ram recorded samples showed that our method reported higher average speed values (χ2= 592.53, Df= 1, p<0.0001 for VCL, χ2= 118.19, Df= 1, p<0.0001 for VSL and χ2= 112.33, Df= 1, p<0.0001 for VAP, Fig. 5A), average AHL (amplitude of the lateral displacement of the head, χ2= 102.55, Df= 1, p<0.0001, Fig. 5B), BCF (wavelengths of the flagellar beat, χ2= 157.09, Df= 1, p<0.0001, Fig. 5B) and also a higher motile population (χ2= 85.17, Df= 1, p<0.0001, Fig. 5C) compared to SCA® reports. Similar results were observed for these kinetic values when buck samples were analyzed (Fig. 5 D-F, χ2= 23.49, Df= 1, p=0.0004 for VCL, χ2= 12.81, Df= 1, p=0.0003 for VSL, χ2= 17.55, Df= 1, p<0.0001 for VAP, χ2= 76.20, Df= 1, p<0.0001 for BCF, χ2= 6.39, Df= 1, p=0.0115 for ALH and χ2= 18.52, Df= 1, p<0.0001 for total motility). Manual validation showed that SCA® failed in tracking and reporting sperm with very high speeds often rendering in non-detected cells (e.g., sperm indicated with yellow arrows in Fig. 3) or otherwise fragmenting the trajectories with low speed assigned to a little stretch (e.g., sperm indicated with yellow arrows in Fig. 6). Accordingly, the report of kinetic values obtained by using both methods over the same cells (i.e. cells tracked by both softwares) showed comparable values (Fig. 6, green arrows). Consequently, differences can be explained on the basis of the better performance of our sperm tracker to report speed parameters for very fast motile sperm and to the increment in the number of tracks (Table 2) mainly corresponding to motile spermatozoa (Fig. 5C and F).

## Discussion

In this work, we presented a new detection and tracking algorithm that can effectively identify immotile as well as motile and progressive sperm heads from two different species, with different concentration ranges and bearing different proportions of motile sperm. We demonstrated that the proposed approach can successfully handle challenges such as cell collision and occlusion, succeeding in multiple sperm tracking, when the spermatozoa concentration up to 42.29 x $10^6$ cells/ml. Our free access tool was validated against CASA SCA® , providing similar values of sperm parameters but was more efficient in the number and precision of detected tracks at high concentration ranges, as well as in relation to the lower number of fragmented trajectories.

Some single particle tracking algorithms have been developed, however they mostly failed in following them simultaneously when more than 10 cells co-exist (Imani *et al.* 2014, Tinevez *et al.* 2017). Recently, an automated multi-sperm tracking algorithm capable to detect and track simultaneously hundreds of human sperm cells from two samples was presented with the limitations of long time required to process each video at low acquisition speed and lack of validation against a standardized method (Urbano *et al.* 2017).

Many cells segmentation methods have been proposed in literature for microscopy image sequences. Some works first binarize the images and others use a matching template.

Hidayatullah *et al.* (2015), Gárate Polar (2015) and Rojas *et al.* (2012) proposed a fixed threshold and then a binary morphological filter; Buchelly *et al.* (2016) applied a mathematical morphology gray filter to highlight sperm heads and a later threshold; Liu *et al.* (2013) , Vinaykumar and Jatoth (2014) applied temporal frame differencing, a fixed threshold and a binary morphological filter and others use background subtraction, thresholding and binary filtering (Azari *et al.* 2011, Jeong *et al.* 2014). Other approaches also exist that use simultaneous detection and tracking with their own considerations (Karthikeyan *et al.* 2012, Boryshpolets *et al.* 2013).

It is consensus that standardization is needed to avoid variations in semen analysis (Palacín *et al.* 2013). One of the most important settings of the assay is cell concentration. In this sense, Wilson-leedy and Ingermann (2007) have studied the effect of the cells concentration upon motility measurements, as well as we do, finding that the main limitation is particle density. The widest dynamic range allowed the higher plasticity of the tool, which is critical when considering working with sperm from different species For example, the VCL range for ram motile sperm is between 189.8 ± 40.7 and 39.8 ± 21.0 μm/s (Ledesma *et al.* 2017), while for fish the VCL range is between 330 ± 70 and 20 ± 15.0 μm/s for a variety of species (Fauvel *et al.* 2010, Fabbrocini *et al.* 2016). This is an advantage when compared to commercial systems that are specie-specific. On the other hand, there should be some other adjustments needed for correct sperm identification in other species associated to differences in sperm morphology and size.

According to Lucena (2003), a typical tracking scheme has four basic essential elements: image features, model of the objects, model of the dynamics, and a temporal fusion method. The most of the works in the state of the art use intensity distribution (Karthikeyan *et al.* 2012, Rojas *et al.* 2012, Jeong *et al.* 2014, Gárate Polar 2015, Hidayatullah *et al.* 2015); however there also exist more features that can be used like color (Lucena *et al.* 2010, Fang *et al.* 2017), motion history (Liu *et al.* 2013), optical flow (Lucena *et al.* 2015), frequency descriptors (Pei *et* *al.* 2006), and others. As mentioned before, we used the intensity distribution as the image feature required to detect the spatial distribution of cells at each instant, because of the high contrast obtained between foreground and background in the scene.

A common trouble in tracking systems is the occlusions handling. In this situation, two or more objects in the 2D scene get very close to each other and the detection module often considers them as a single object, giving to the system the ambiguity of which label corresponds to this new object and how to treat the absence of the missing others. The tracking scheme must lead with this situation and take a proper decision. The method (Azari *et al.* 2011) applied template matching in the region of occlusion and used correlation to identify the parts corresponding to each merging individual object. In Jeong *et al.* (2014) the aspect ratio or width/height is considered to detect when object are merging or splitting. In Lucena *et al.* (2010), authors use a combined model of the mean-shift and the CAM-Shift algorithms to improve robustness to occlusion. The occlusion handler of Sahbani and Adiprawita (2016) uses the statistics of the blob size (standard deviation) to find an occlusion situation by means of an occlusion threshold. When an occlusion condition occurs due to merging objects, the label of the new particle corresponds to the label of the previous object that presented the closer prediction point to the measured mass center. Meanwhile, the position of the hidden object is predicted during a test interval of 6 frames with an increasing search radius. Then, if the particle appears during the test interval and inside the search region, it will recover its original label and its path will be completed with the previous estimated locations.

Many similar works have developed solutions to make an automatic motility analysis, both for human and other animals' sperm. In this work, we take the known methods to determine the motility parameters and own considerations, but we put our major interest in the system performance evaluation. An important fact to consider is the objectivity of the curvilinear velocity (VCL) measurement and the subjectivity of the average path velocity (VAP). The VAP parameter depends of the smoothness degree of the spermatozoon trajectory and there is no information about a unified criterion to perform that low pass filtering operation and, in consequence, each system performs it in a different way giving probably different results for the same sample video. The subjectivity in the method to measure the VAP parameter also carries subjectivity in measuring the other ones that depends on it: Amplitude of Lateral Head Displacement (ALH) and Beat Cross Frequency (BCF).

By the other hand, the VAP calculation depends on the particular method used for obtaining the smoothed path. In Hidayatullah et al. (2015), authors showed the implementation of a moving average filter with a fixed size of 5 elements. Rojas *et al.* (2012) used an approximation based on the Bezier Plane method. Wilson-Leedy and Ingermann (2007) used a moving average filter which size depends on the frame rate. The Microptic's - SCA® establishes VAP as one of the modifiable parameters by the user and thus makes it more inter subjective.

Sperm kinetic parameters determined by our software compared with the values offered by the reference software (Microptic's - SCA®) over the same samples were able to get comparable output data when measuring the same sperm particles. However due to the better performance of our software to correctly track high speed sperm, a higher percentage of rapid sperm and consequently average higher speed values were reported by our algorithm. It is important to consider that the measure of VSL depends only on the final and initial points of each path, so it could be directly validated by the tracking performance. Moreover, for different samples and laboratories comparisons between available CASA systems should be carefully done since several factors inherent to motility acquisition settings affect the standardization. The other parameters (VAP, ALH, BCF) depend on which smooth filter was applied to the original path and currently there is no standardized criterion to select one as the best choice, as mentioned before.

Finally, whereas most of the studies conducted nowadays to boost standardization of sperm motility assessment systems are focused on the software capacities, in this study we also analyze the equipment requirements. It is known that commercial systems have been improving their software and also associated cameras according to users' demand. However, in the research field labs acquire commercial CASA systems that cannot be often modernized and furthermore, publications are based on available equipment. In this sense, the choice of the velocity parameter describing the motility also depends on the video camera used.

According to Wilson-leedy and Ingermann (2007) , low speed recording will hide the modifications of tracks during large time intervals (1/25 s for example) so that VCL and VAP would be quite similar. Our tool can be adapted to a range of acquisition speed (fps), suggesting that the tracking system could manage different number of frames in the same time lapse.  This is particularly useful for species with high speed sperm, complex trajectories or unusual head/flagella movements.

As a conclusion, this work presented new an-open-source sperm tracker software to sperm motility analyze at a range of different cell concentrations, taking ram and buck sperm as models. The tool has the possibility to be adapted by the creators to any other sperm species.

**Declaration of interest**

The authors have nothing to disclose.

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**Legends and Tables**

**Sperm tracking videos.** Each line indicates the spermatozoa tracked by our sperm tracker software frame to frame. Numbers identify each spermatozoa. Different colors of paths indicate the different sperm velocities (static, low, medium or rapid sperm).

**Figure 1: Signal processing for the detection process. Upper panel**, a region of interest in a sample frame is selected to explain the detection process. **Lower panel,** Top-Hat transformation of the selected area (left), binary image obtained by applying a fixed threshold (center), and binary morphological opening to obtain only the heads(right). Bar= 25 µm.

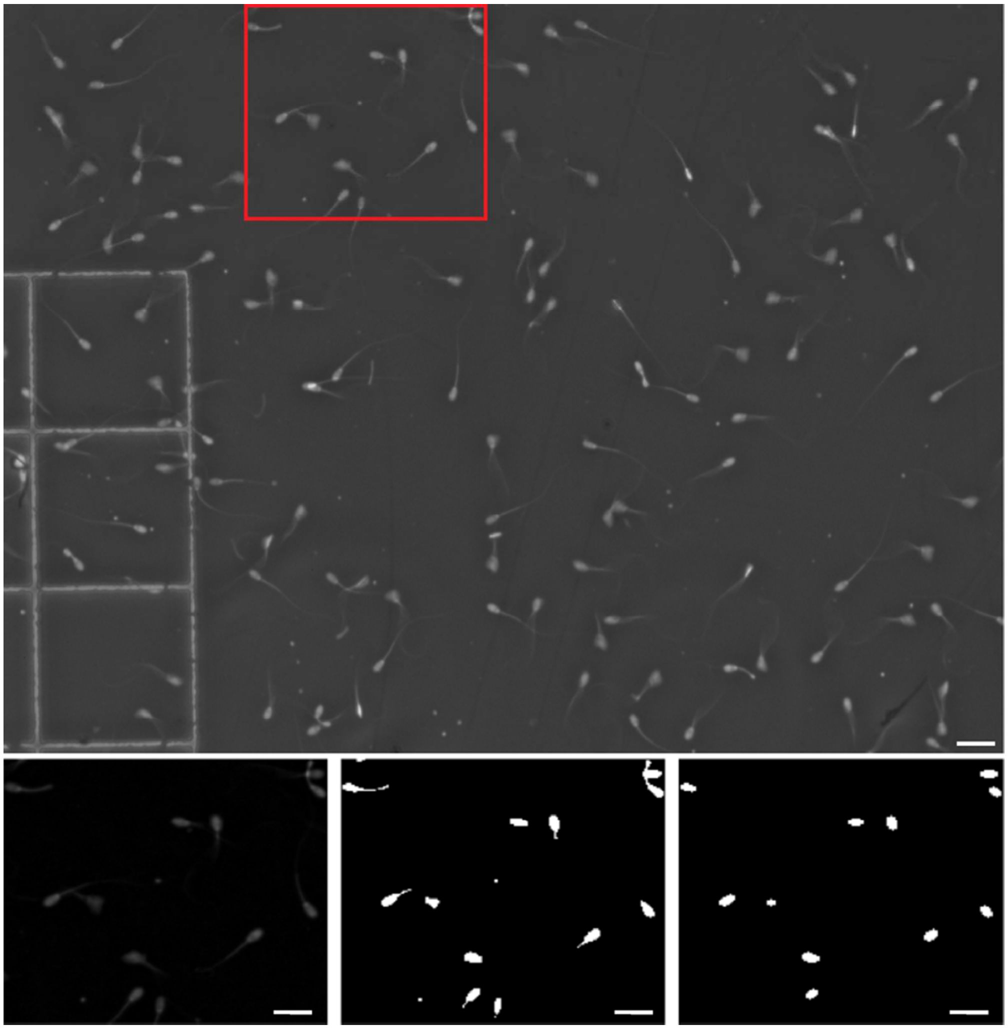
**Figure 2: Zoom of a region with detected spermatozoa heads and their centroids.** Bar= 10 µm.

**Figure 3: Visual comparison between trajectories detected by the SCA® versus the trajectories detected by the purposed sperm tracker software**. The images correspond to the same video acquired at 25 fps (Video 5 of Table 2, https://vimeo.com/264485794), showing the totality of paths detected by the SCA® module **(upper panel)** or our tracker software **(lower panel).** Bar= 25 µm. Curvilinear Velocity (VCL, µm/s) of some sperm that were not detected with SCA® software (yellow arrows) are indicated with labels and arrows in the bottom panel corresponding to our software.

**Figure 4: Performance of the sperm tracker software.** Percentage of fragmented paths **(A, B and C)** or precision **(D, E and F)** for our Sperm tracker system (SMT software) compared to SCA® (SCA software) at 25 fps. Ram (A and D) or buck (B and E) sperm were analyzed. **C and F panels** gather results of all the analysis consider cell concentration as a variable. Data assemble the reports of three independent experts that analyzed 10 videos for each species. * Value significantly different with respect to SCA ®software ($p < 0.05$).

**Figure 5: Report of Kinetic parameters analyzed by the commercial CASA system (SCA®) and the sperm motility tracker software (SMT) purposed by us.** Sperm velocities: VAP, VSL and VCL (**A and D**), ALH and BCF (**B and E**) as well as total motility (**C and F**) were analyzed by both methods (SCA and SMT software) over the same samples. Analysis of ram samples are shown in A-C whereas analysis of buck samples are shown in D-E. . * Value significantly different with respect to SCA® software ($p < 0.05$). VAP: Average Path Velocity, VSL: Straight-Line Velocity, VCL: Curvilinear Velocity, ALH: Amplitude of Lateral Head Displacement, BCF: Beat Cross Frequency.

**Figure 6: Visual comparison between trajectories detected by the SCA® (upper panel) versus the trajectories detected by the purposed sperm tracker software (bottom panel)**. The images correspond to the same video (Video 2 of Table 2, https://vimeo.com/264482322) acquired with 25 fps, showing the totality of paths detected with the SCA® module (upper panel) and our tracker software (bottom panel). Bar= 25 µm. Curvilinear Velocities (VCL, µm/s) of some paths fragmented by SCA® software but tracked correctly by SMT are indicated with labels (1-9) and yellow arrows in the corresponding panels. Tracks with similar VCL (µm/s) comparing SMT to SCA® are indicated with labels (10-13) and green arrows in the corresponding panels.



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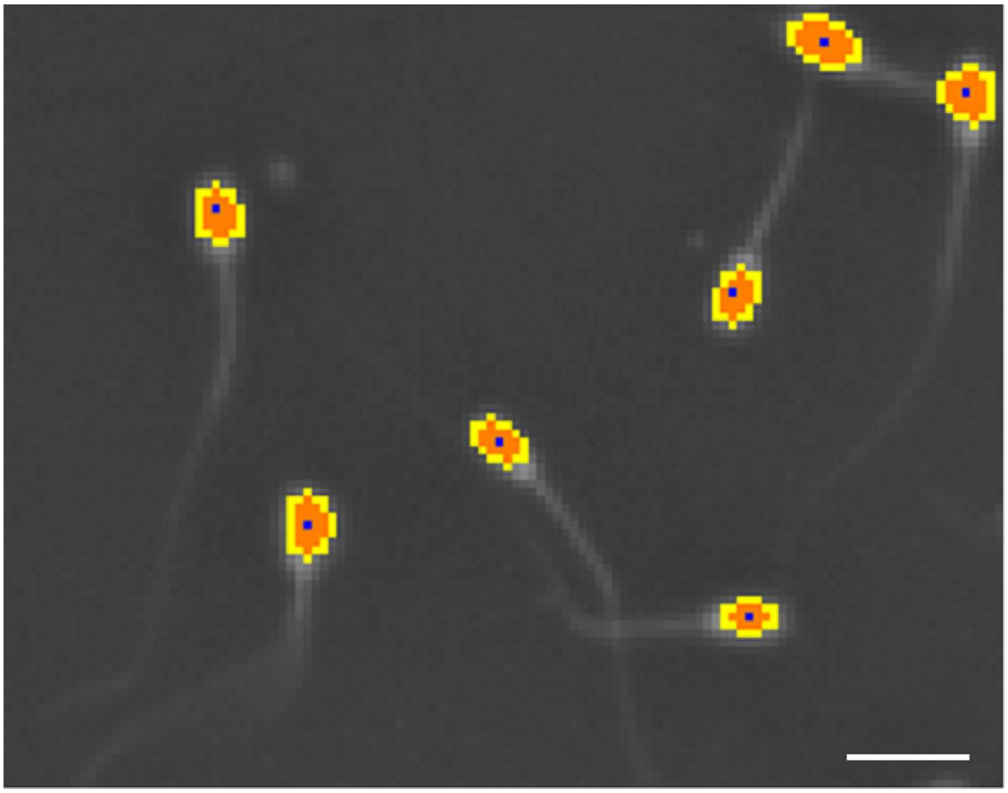

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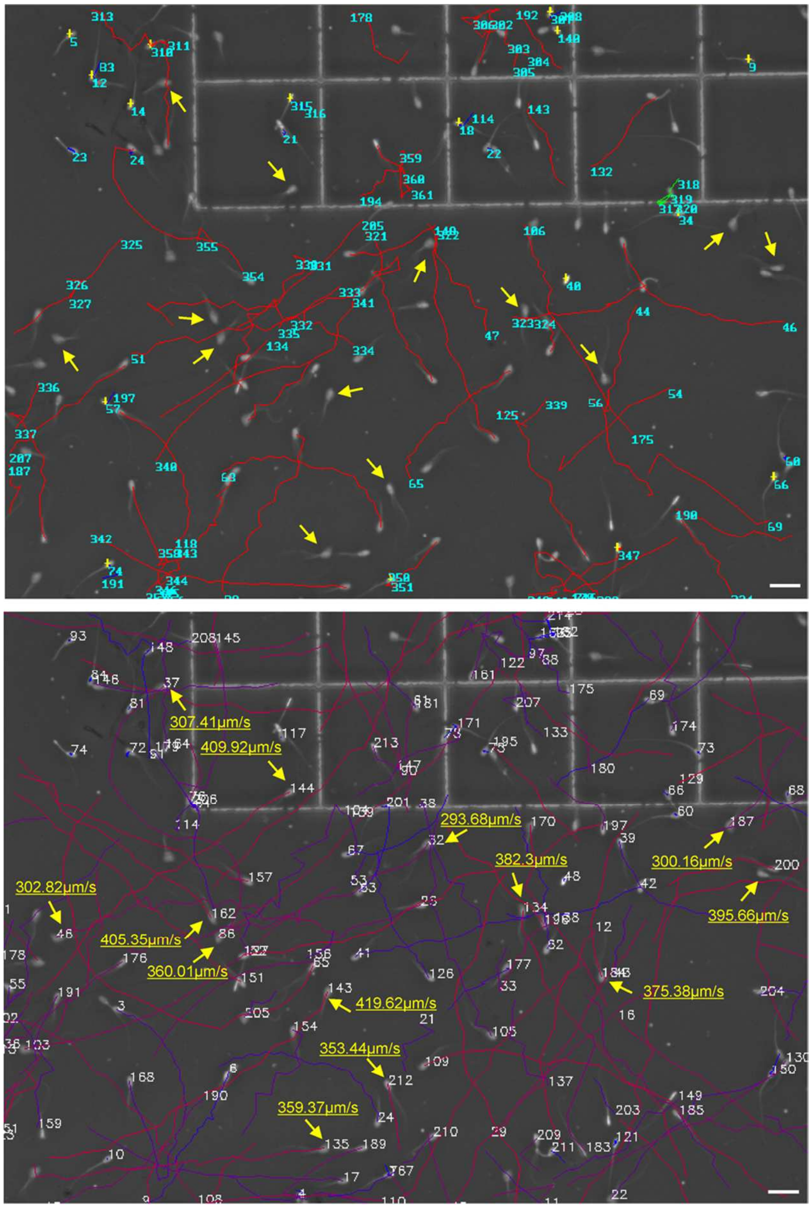


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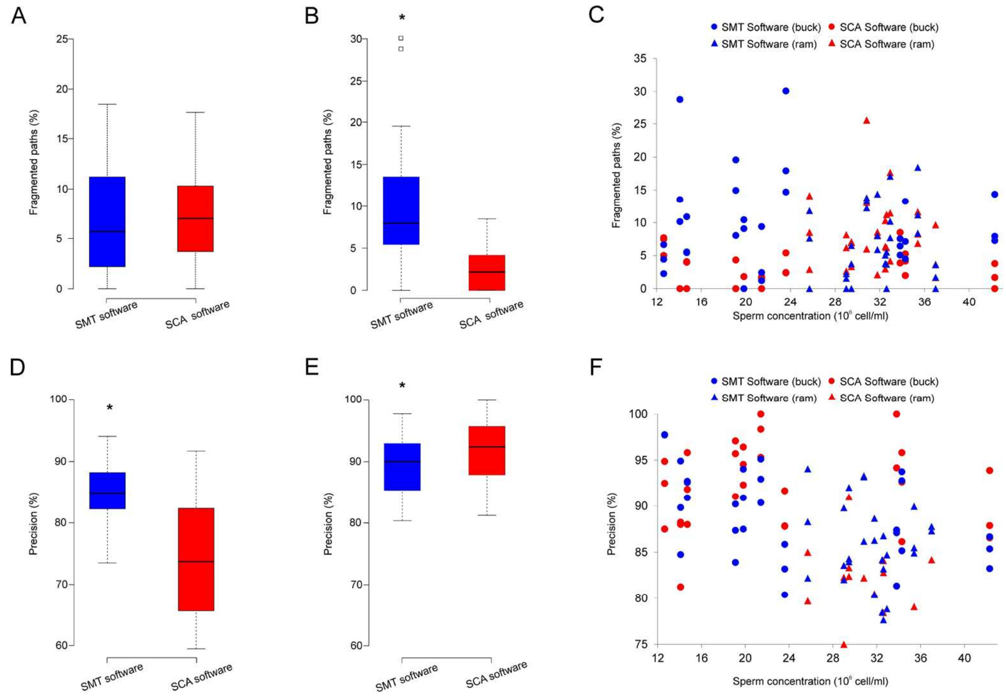


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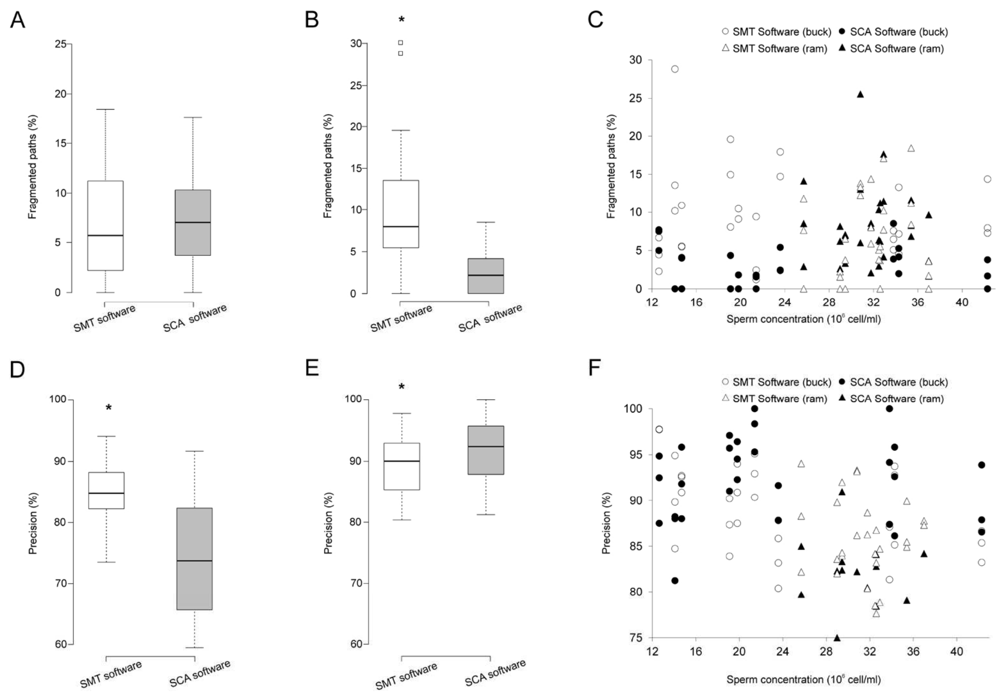


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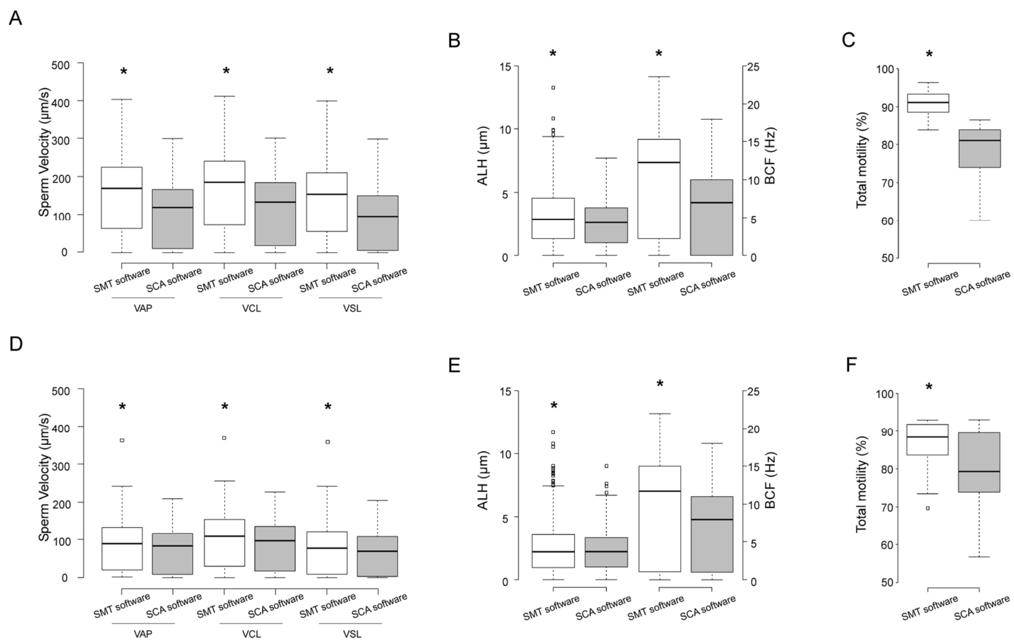


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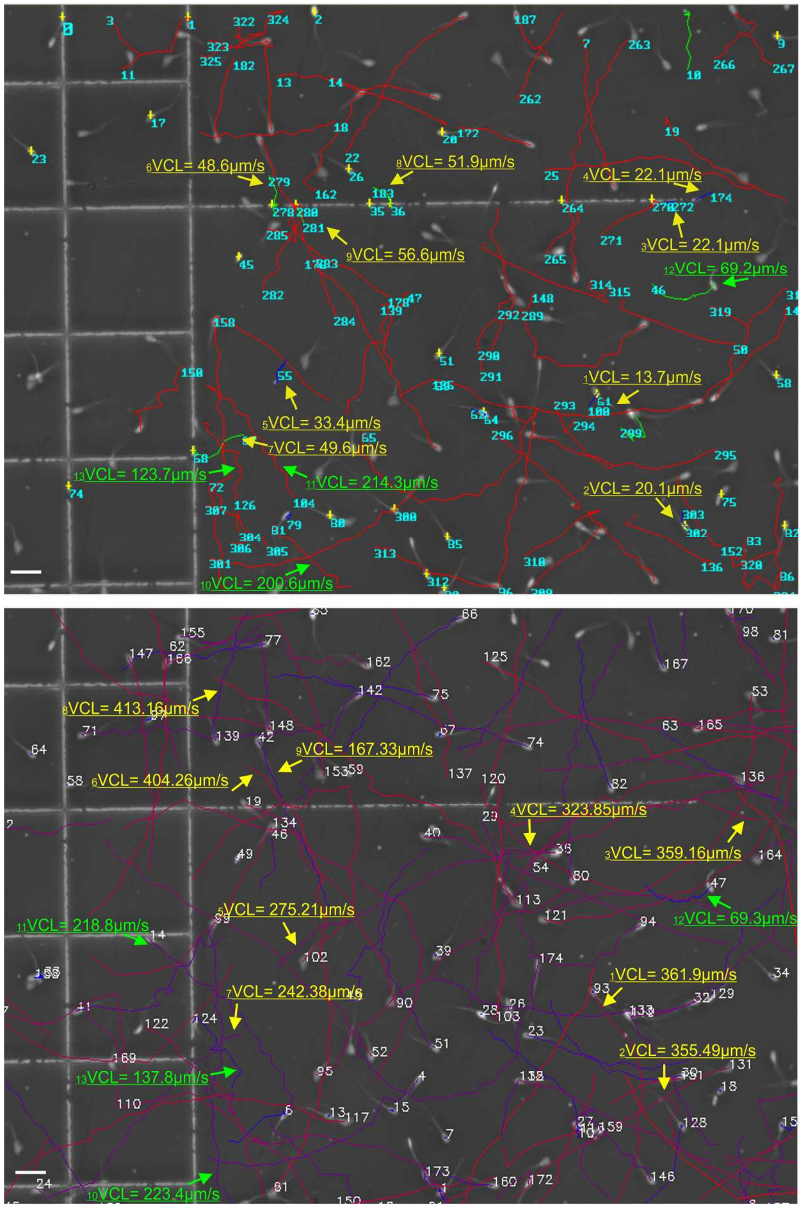


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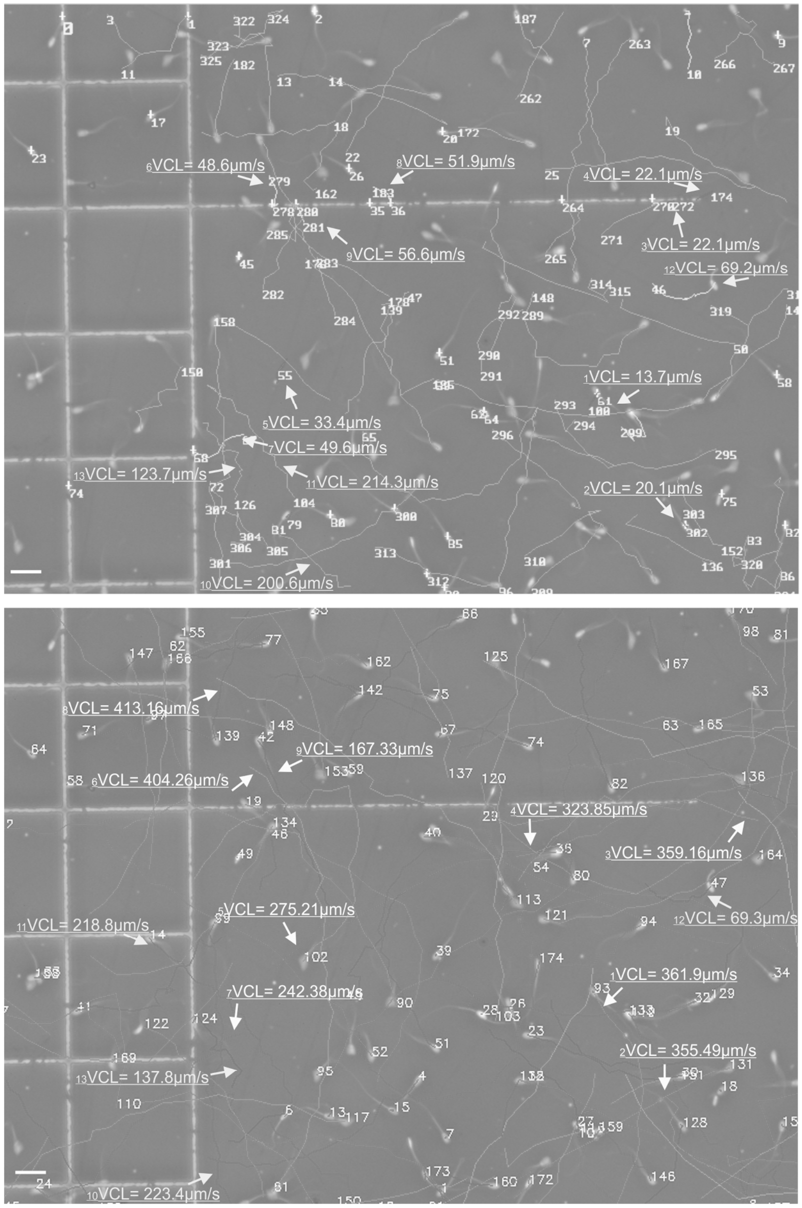


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170x256mm (300 x 300 DPI)

**Table 1. Requirements for motility acquisition and analysis**

| Parameter | Characteristics |
|---|---|
| Chamber depth | 10 µm |
| Maximum number of cells per field | ≤ 120 |
| Optimal sperm concentration | ~ $35*10^6$ cells/ml |
| Acquisition frame velocity with the camera | ≥ 25 fps/sec* |
| Video recording time | 5 sec |
| Microscope setting | Phase contrast, 10 x |
| Input to the software | Sequence of time-lapse images (MP4, AVI or MOV video) |
| System requirements to run software | I3 processor, 3 GB RAM, 1280x800 screen resolution. |

* In this work, the kinetic values were compared between methods for 25 fps/sec due to camera limitations.

**Table 2. Performance of the proposed tracker software (SMT) compared to SCA system**

| | | | Software Proposed Method | | | | | SCA® system | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Animal** | **Nº video** | **Concentration ($10^6$ cells/ml)** | **% SCA tracks followed by SMT** | **Evaluated tracks +SE** | **% correctly detected particles (over total labelled particles)** | **Fragmented paths +SE (%)** | **Precision +SE (%)** | **Evaluated tracks +SE** | **% correctly detected particles (over total labelled particles)** | **Fragmented paths +SE (%)** | **Precision +SE (%)** |
| Ram | 1 | 32.49 | 76.4 + 10.1 | 158 + 0 | 100 + 0.0 | 4.2 + 0.7 | 82.3 + 3.3 | 102 + 5 | 84.0 + 4.2 | 6.5 + 3.7 | 66.1 + 3.5 |
| | 2 | 28.99 | 81.1 + 14.5 | 131 + 8 | 93.1 + 5.4 | 1.3 + 1.1 | 85.1 + 4.1 | 84 + 14 | 74.3 + 12.3 | 5.7 + 2.8 | 76.9 + 4.8 |
| | 3 | 36.99 | 81.0 + 11.9 | 165 + 3 | 89.7 + 1.6 | 1.8 + 1.8 | 82.8 + 8.1 | 93 + 29 | 73.5 + 22.8 | 5.0 + 4.2 | 74.3 + 8.9 |
| | 4 | 32.60 | 78.2 + 14.2 | 155 + 19 | 88.4 + 10.6 | 3.1 + 2.8 | 82.5 + 4.6 | 84 + 30 | 64.9 + 23.0 | 5.8 + 5.6 | 81.8 + 3.0 |
| | 5 | 29.45 | 83.6 + 9.8 | 151 + 11 | 89.9 + 6.8 | 3.4 + 3.3 | 86.8 + 4.6 | 72 + 14 | 71.6 + 13.5 | 5.8 + 2.1 | 85.9 + 4.8 |
| | 6 | 35.40 | 79.4 + 1.3 | 179 + 1 | 99.6 + 0.3 | 12.7 + 5.2 | 86.8 + 2.8 | 108 + 19 | 82.2 + 14.4 | 8.9 + 2.5 | 69.2 + 9.6 |
| | 7 | 31.80 | 74.3 + 3.5 | 152 + 2 | 98.7 + 1.1 | 9.4 + 4.4 | 85.1 + 4.3 | 95 + 14 | 79.4 + 11.7 | 6.2 + 3.6 | 67.8 + 11.2 |
| | 8 | 32.92 | 82.0 + 4.9 | 157 + 1 | 98.1 + 0.6 | 11.7 + 4.9 | 79.4 + 5.0 | 92 + 6 | 76.0 + 5.0 | 11.1 + 6.7 | 69.1 + 3.7 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 9 | 30.82 | 80.9 ± 2.4 | 150 ± 3 | 98.5 ± 1.7 | 13.1 ± 0.8 | 90.9 ± 4.1 | 99 ± 11 | 82.9 ± 9.3 | 14.9 ± 9.9 | 68.0 ± 12.4 |
| | 10 | 25.70 | 88.4 ± 10.7 | 118 ± 3 | 85.3 ± 1.8 | 6.5 ± 6.0 | 88.2 ± 5.9 | 74 ± 4 | 78.7 ± 4.6 | 8.5 ± 5.6 | 79.6 ± 5.5 |
| **Buck** | 1 | 19.82 | 98.2 ± 0.1 | 65 ± 2 | 82.7 ± 2.6 | 6.5 ± 5.7 | 90.8 ± 3.3 | 54 ± 2 | 92.1 ± 3.5 | 0.6 ± 1.0 | 94.4 ± 2.1 |
| | 2 | 21.41 | 99.5 ± 0.9 | 83 ± 2 | 96.51 ± 2.0 | 4.4 ± 4.4 | 92.8 ± 2.4 | 60 ± 4 | 90.0 ± 6.0 | 1.1 ± 1.0 | 97.9 ± 2.4 |
| | 3 | 42.29 | 97.1 ± 0.3 | 165 ± 2 | 98.2 ± 1.2 | 9.9 ± 3.9 | 85.1 ± 1.7 | 127 ± 7 | 92.0 ± 5.1 | 1.8 ± 1.9 | 89.4 ± 3.9 |
| | 4 | 33.81 | 97.1 ± 0.9 | 123 ± 6 | 93.2 ± 4.2 | 6.4 ± 1.2 | 85.3 ± 3.4 | 102 ± 2 | 96.8 ± 2.2 | 5.4 ± 2.7 | 93.9 ± 6.3 |
| | 5 | 34.28 | 99.3 ± 1.1 | 114 ± 5 | 93.2 ± 3.9 | 8.3 ± 4.5 | 90.6 ± 4.7 | 97 ± 4 | 92.9 ± 3.9 | 3.8 ± 1.7 | 91.5 ± 4.9 |
| | 6 | 12.64 | 100 ± 0.0 | 44 ± 1 | 98.5 ± 1.3 | 4.5 ± 2.2 | 97.8 ± 0.0 | 40 ± 1 | 96.7 ± 1.4 | 6.7 ± 1.5 | 91.6 ± 3.8 |
| | 7 | 14.7 | 95.2 ± 1.3 | 54 ± 1 | 98.8 ± 1.0 | 7.3 ± 3.1 | 92.1 ± 1.0 | 49 ± 1 | 94.2 ± 1.9 | 2.7 ± 2.3 | 91.9 ± 3.9 |
| | 8 | 14.09 | 95.3 ± 1.1 | 59 ± 1 | 99.4 ± 1.0 | 17.5 ± 9.9 | 89.8 ± 5.1 | 50 ± 2 | 92.0 ± 2.8 | 0.0 ± 0.0 | 85.8 ± 4.0 |
| | 9 | 19.1 | 97.1 ± 2.6 | 88 ± 3 | 93.4 ± 3.4 | 14.2 ± 5.8 | 87.2 ± 3.2 | 69 ± 2 | 95.4 ± 2.1 | 1.4 ± 2.5 | 94.6 ± 3.2 |
| | 10 | 23.59 | 97.5 ± 1.1 | 107 ± 6 | 93.6 ± 5.3 | 20.9 ± 8.1 | 83.1 ± 2.7 | 80 ± 6 | 89.5 ± 6.6 | 3.4 ± 1.7 | 89.1 ± 2.2 |