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Advanced Semen Evaluation Techniques: Computer Assisted Sperm Analysis (CASA) and Flow Cytometery: Review

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Abstract

The poor rate of success for artificial insemination (AI) can be attributed to many intrinsic and non-intrinsic causes like the individual animal's semen quality, the minimum standard set for semen quality prior to acceptance for freezing and post-thawed semen, the freezing and thawing protocol and the number of spermatozoa per straw for insemination. And even though, a wide variety of evaluation methods and/or tools are used to investigate the quality of bull semen, it is still difficult to determine to which extent subfertile sperm contributes to the inability of achieving conception. However, analysis of semen for an individual bull based on different parameters provides better information to predict its fertility. For this reason; now a days semen production centers are moving away from subjective semen assessment which is largely uncorrelated to field fertility to objective semen analyses that incorporate computer assisted sperm analysis (CASA) and flow cytometry. A multiparametric approach to semen analysis using CASA, flow cytometry and preferably in combination; can make possible the semen production centers to produce high quality semen. In this paper I reviewed these two advanced semen evaluation methods (CASA and flow cytometery) for their

multiparameteric semen quality evaluation approach (sperm motility, kinematics, viability, acrosomal integrity, mitochondrial activity, DNA integrity) and the possible factors and/or limitations affecting the results of the technologies when they are used for analysis of spermatozoa.

Keywords: Computer Assisted Sperm Analysis, flow cytometry, Semen analysis.

Introduction

Artificial insemination is already a proven bio technique and has several advantages over natural mating as it helps in harnessing the genetic potential of a bull to its maxima (Brito, 2010). The poor rate of success for AI can be attributed to many intrinsic and non-intrinsic causes; some of which from the genetic material side of the bull are: the individual animal's quality of semen produced, the minimum standard set for semen quality prior to acceptance for freezing and post-thawed semen, the freezing and thawing protocol and the number of spermatozoa per straw for insemination (Lemma, 2011). In the artificial insemination industry; the semen produced from high genetic value animals should be well characterized and handled to optimize its quality (Vincent *et al.,* 2012).

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Appropriate evaluation of the semen for artificial insemination has significant importance in livestock sector. Even though, a wide variety of evaluation methods and/or tools are used to investigate the quality of bull semen, it is still difficult to determine to which extent subfertile sperm contributes to the inability of achieving conception (Muhammad *et al.,* 2013).

The complexity of sperm cell structure and subjectivity of conventional semen analysis methods lead to difficulty to obtain precise and accurate results. Conventional sperm motility estimation is done by visual approximation of progressively moving spermatozoa using phase contrast microscope; for this reason it lacks the ability to measure the sperm functional status, kinematics and is also subjective depending on the experience of the person who performs the analysis. As a result; it limits the prediction for fertilizing potential of male animals (Amann, 1989). Analysis of semen for an individual bull based on different parameters provides better information to predict its fertility (Madeja *et al.*, 2003). Advanced semen evaluation techniques like Computer-assisted semen analysis (CASA) and flow cytometery provide a solution by reducing technical variability for the problems which could be developed from

subjective conventional semen analysis (Bochenek *et al*., 2001). With the help of CASA motility and kinematic parameters can be measured in an objective manner. These enhance the accuracy of semen motility assessment and are also beneficial for its capacity to yield repeatable and highly reliable results on kinematics of ejaculates based on measurements of individual sperm cells *(Agnieszka et al., 2012)*. Flow cytometry is used to analyze a variety of structural and functional characteristics of spermatozoa such as plasma membrane integrity, chromatin structure, mitochondrial membrane potential, acrosome integrity, changes in the sperm surface induced by sperm capacitation, and certain forms of morphological abnormalities present in a sperm sample through relative fluorescent intensity (Bochenek *et al*., 2001). Hence, this review is aimed to review the application of advanced multiparameteric semen evaluation techniques (CASA and Flow cytometery) in predicting fertility potential of bulls.

Advanced Semen Evaluation Techniques

Sperm cell comprises complex structures contributing more for the successful fertilization of the ovum (Madhuri *et al.,* 2012). In addition to the complexity of sperm cell structure; it is also difficult to obtain precise and accurate results using traditional subjective semen analysis methods. On the other hand, a number of studies have shown that technical variability can be reduced when objective methods are used (Katz and Dott, 1975; Ketz and Overstreet, 1981; Bochenek *et al*., 2001; Vincent *et al.,* 2012). These methods also provide other measures of spermatozoa behavior like individual cell kinematics, structures and functions which are important to predict the fertility potential of individual bull. Now a days automated machines such as computer assisted sperm analyzer (CASA) and flow cytometer are developed to evaluate various characteristics of a sperm cell such as motility and morphology parameters, internal compartments and their functions at individual cell level (Agnieszka *et al.,* 2012).

Computer Assisted Sperm Analyzer (CASA)

Computer assisted sperm analysis is a powerful tool for the objective assessment of sperm motility and hence now is one of the choice of techniques for evaluating semen quality (WHO, 2010). The basic components of this technology are a microscope to visualize the sample, a digital camera to capture images and a computer with its specialized software to analyze the movement of the spermatozoa (Vincent *et al.,* 2012). This method was first

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developed using multiple time-exposure photomicrographies to follow spermatozoid movements (Madhuri *et al*., 2012). The essential principle behind microscopy-based CASA systems is that a series of successive images of motile spermatozoa within a static field of view are acquired by computer software algorithms, which then scan the image sequences to identify individual spermatozoa and trace their progression across the field of view. This involves recognizing the same cell in each image by its position, and inferring its next position by estimating the likelihood that it will only have moved a certain maximum distance between frames (Vincent *et al.,* 2012). This automated system is designed to provide precise and meaningful information about sperm concentration, viability, dynamics or morphology, and to perform the statistical analysis of sperm population based on the development of the continuous images of spermatozoa, digital processing and information analysis with the aids of its video camera, video capture card and computer (Lu *et al.,* 2013).

As different factors and/or features affect CASA results; such features should be addressed with appropriate laboratory protocols and quality control procedures. Therefore; while using CASA following guidelines which can enable the workers to obtain accurate information without encountering technical limitations is necessary. For example as it is summarized in Annex 1, laboratory supplies can be routinely screened for cytotoxicity, the effect of temperature on spermatozoon motility can be controlled by using a heated microscope stage, Liquate variation can be reduced by using standardized protocols for specimen preparation with appropriate quality control procedures, variability in counting chamber can be reduced by using one of the new disposable sperm counting chamber, the accuracy of kinematic parameters particularly VCL, BCF and ALH can be improved by using a video framing rate that is suitable for the physiological state of the sperm, the stability of kinematic parameters can be improved by tracking all sperm for sufficient number of video frames, statistical biases can be eliminated when summery statistics are computed on sperm trajectories of equal length and the accuracy of population estimates can be increased if sufficient number of motile sperm are analyzed.

Description of CASA motility parameters

As described here in the underneath percent motility, progressive motility, curvilinear velocity, straight line velocity, amplitude of lateral head displacement, Linearity, average path

velocity, straightness and beat cross frequency are some of the major motility parameters assessed by CASA system (Niżański *et al*., 2009; Agnieszka *et al.,* 2012).

Percent motility (MOT): According to Madhuri *et al*. (2012) sperm motility percentage is defined as the number of motile cells divided by the total number of cells analyzed and expressed in percent. A cell is considered to be motile if its average straight line speed (VSL) met or exceeded the minimum motile speed parameter. According to Sundararaman *et al.* (2012) the minimum motile speed parameter for bull sperm is 4.4μm/s. For analysis, at least a total of 200 cells should be analyzed to express the percentage of motile cells (Madhuri *et al*., 2012).

Progressive motility (PMOT): Progressive motility is defined as the populations of cells that are moving actively forward and is expressed in percentage (Agnieszka *et al.,* 2012). According to Paul (2013) a progressively motile sperm is defined as the one which has average path velocity (VAP) > 50 µm/s and a straightness ratio (STR) > 75%.

Straight line velocity (VSL): This is measured in µm/s and defined as the average velocity measured in a straight line from the beginning to the end of the track. It is a measure of cell's forward progression and is computed by multiplying the curvilinear velocity (VCL) with mean linearity and divided by 100. It is computed for the population of motile cells by averaging the mean values of individual cell (Madhuri *et al.*, 2012).

Curvilinear velocity (VCL): This is computed as the average scalar velocity (or speed) for all motile paths. It is calculated by computing the total distance travelled along each path divided by the time interval. Like that of VSL; population VCL is computed only for motile cells and is achieved by averaging the mean values from each individual cell. According to (Ulfina *et al*., 2014)) it is also measured in μm/s and defined as time-average velocity of a sperm head along its actual curvilinear path as perceived in two dimensions under the microscope.

Velocity of the average path (VAP): It is also measured in μm/s and defined as the average velocity over the smoothed cell path. It is computed by smoothing the actual path and used to characterize the overall trajectory of the sperm cell (Agnieszka *et al.,* 2012; Ulfina *et al*., 2014).

Mean linearity (LIN): The distance a sperm cell travels along its normal (or un-smoothed) path is referred to as its gross displacement. The straight line distance from its starting point to its current X-Y position (as the crow flies) is referred to as net displacement. The ratio of these two measures multiplied by 100 (i.e VSL*100/VCL) is the linearity measure for the spermatozoon. It is evaluated at the end of each of the motile paths and all of the motile cell path values are averaged to form the single number for the report. A cell that swim in a straight line has value of 100 and a cell that had just completed a circle had an instantaneous value of zero (Ulfina *et al*., 2014).

Straightness (STR): Straightness is a measure of VCL side to side movement and determined by the ratio VSL/VAP multiplied by 100. It is also measured in percentage (Agnieszka *et al.,* 2012).

Amplitude of lateral head displacement (ALH): For each cell, the distances between the actual curvilinear and the smooth (or average) path are computed. These values are sometimes referred to as RISERS. This parameter (ALH) is computed by the maximum value of the RISER for each path and then computed as the average value of all of the individual maxima as the single value to include in the report of lateral head displacement of motile cell population (Madhuri *et al*., 2012). According to Agnieszka *et al.* (2012) this parameter is measured in µm and defined as the mean width of the head oscillation as the sperm cell moves. Different CASA instruments compute ALH using different algorithms, so values may not be comparable among systems (WHO, 2010).

Beat cross frequency (BCF): It is measured in Hz and defined as the average rate at which the sperm's curvilinear path crosses its average path (Madhuri *et al*., 2012). Agnieszka *et al.,* (2012) also define this parameter as the frequency with which the sperm head moves back and forth in its track across the cell path.

There are also other ways of expression for motility parameters of spermatozoa like RAP, MED, SLOW and STATIC those expressing subpopulation of cells with (rapid, medium and slow) moving cells and Static cells respectively. And all these expressed in percent (Agnieszka *et al.,* 2012).

Attributes measured by CASA

Motility: Motility is one of the most important characteristics believed to be associated with the fertilizing ability of spermatozoa. A significant correlation between total (Correa *et al*., 1997; Gillan *et al*., 2008) and progressive (Kathiravan *et al*., 2008) motility of bull semen and its field fertility have been previously reported. However, as it was described by some authors, conventional analysis of semen motility did not correlate with fertility (Farrell *et al*., 1998; Januskauskas *et al*., 1999). CASA instruments collect a wide range of sperm motility parameters, allowing a more detailed and accurate analysis of sperm movements and track speeds. Researchers have also tried to correlate the kinetic parameters with the field fertility of semen. Some studies showed a positive correlation between straight line velocity of spermatozoa and field fertility (Farrell *et al*., 1998; Januskauskas *et al*., 1999; Gillan *et al*., 2008; Kathiravan *et al*., 2008); However as it was described by different studies (Davis and Katz, 1993; Farrell *et al*., 1998; Ahmada *et al.,* 2003; Madhuri *et al*., 2012) a very high correlation of bull fertility can be reached by combination of several motility parameter evaluations.

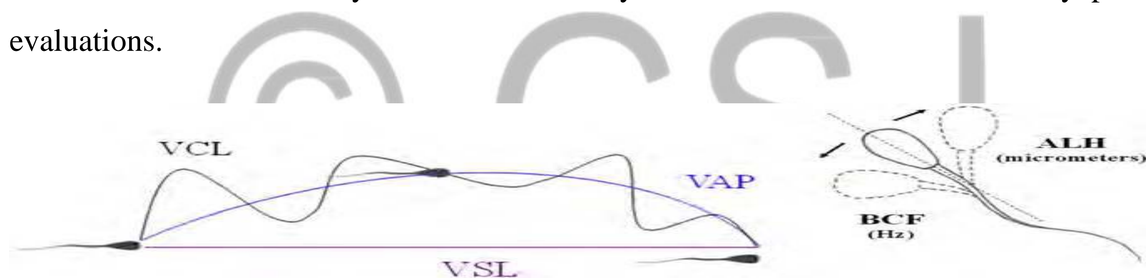

Figure 1: Schemes of different velocities and parameters of sperm movements measured by CASA system. (Source: Agnieszka *et al.,* 2012)

Morphology: On account of the fact that freezing and thawing process provokes morphological or biochemical cryogenic damage; sperm dysfunction and changes in cell's membrane are the expected sequels. The sperm morphology evaluation is an essential component of any semen analysis and provides the clinical information about the potential fertility of semen sample. Mainly morphology of the sperm head is an important criterion for the correct diagnosis of sperm quality (Ahmada *et al.,* 2003).

Though a number of stain methods have been suggested for sperm morphology assessment; they are certainly species-dependent and do not necessarily provide the appropriate gray-level contrast for accurate computer-assisted morphometric analysis. Papanicolaou's staining and

haematoxylin are mostly used for morphologic assessment in CASA (Davis and Katz 1993). This idea is also supported by Ageep *et al.* (2009) who used six types of stains (Haematoxylin, Papanicolaou, Supravital, MGG, Giemsa and Leishman) and interpreted the results of the staining procedure as excellent, very good, good, bad and very bad for each part of the sperm and the cells themselves in the background. And he found best result (excellent and very good of a cumulative percentage more than 86%) for head morphology in these two (Papanicolaou and Haematoxylin) stains.

According to WHO (2010), the CASA software set up can also analyze images of sperm smears stained with Diff-Quik stain. The Diff-Quik dye stained pale blue in the acrosomal region and dark blue in the post-acrosomal region of sperm cell which is a good base for precise image analysis. As a result, by using CASA; morphology of the head parameters such as area of the head, perimeter of the head, brightness, Elng (elongation of the sperm head), FFC(form factor circle- the degree of similarity of the sperm head to a circle), ELL_B (Big axis of ellipse- outlining the sperm head, the length of the sperm head), ELL_S (Small axis of ellipse- outlining the sperm head, the width of the sperm head) and FFE (form factor ellipse- the degree of similarity of the sperm head to an ellipse) can be assessed.

Viability: CASA is also important in identifying sperm viability and differentiation of sperm cells from debris materials under the application of different stain dyes. It determines the sperm viability by using a vital stain- VIADENT'. This dye stains only the cells with non-intact membranes; thus identifying non-viable from viable cells. CASA also differentiates sperm cells from debris using IDENT stain under fluorescent illumination. IDENT is a DNA specific dye based on Hoechst bisbenzimide and stains all DNA containing objects which can fluoresce under appropriate light (Madhuri *et al*., 2012). Cytoplasmic detritus, which is devoid of DNA material, have lower degree of fluorescent intensity than the haploid sperm and may not fluoresce. Interestingly, although sperm cells have only half the DNA complement of a somatic cell, it is highly condensed, resulting in a higher degree of fluorescent intensity (Contri *et al*., 2010)

Biological results obtained by CASA

Keshava (1996) reported a mean motility of 65.22% for fresh semen of Sahiwal breed. His study also showed as low as 39.4% motility for Freiswal and as high as 86.2% in Karan Freis

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(KF) cross breed dairy bulls. Motility was known to be 78.49% for fresh semen of Sahiwal bulls by Ulfina *et al*. (2014). Though not significant, their finding revealed higher motility percentage for older bulls (79.49%) and larger SC (82.10%) than younger bulls (77.12%) and smaller SC (77.12%). The authors also reported a mean post thaw percent motility of 69.62 and 61% at 0 and 24 hr after freezing respectively for Sahiwal bulls. In contrary Muhammad *et al.* (2010) reported lower post thawed percentage of sperm motility (50.6% at 0 hr, 33.8% at 2 hr and 18.1% at 4 hr) for epididymal spermatozoa of Sahiwal breed bulls. This low post thawed percentage of sperm motility might be attributed to less matured epididymal spermatozoa. Similarly Lenz *et al*. (2010) reported a low post thaw motility percentage of 36.8 and 35.1 by using 2-cell leja slide and 4-cell leja slide respectively. The authors also reported a mean progressive motility percentage of 17.3 and 15.1% for the respective leja slides.

Vantman *et al.* (1988) measured the dependence of VSL upon sperm concentration and found an inverse relationship that was caused by the optical conjunction of sperm images when swimming paths crossed. Fast moving sperms are more likely to collide with other cells and also might cross the field of view before analysis is conducted; for these reasons they will be excluded from the analysis and thus lowering the value for VSL. Ulfina *et al.* (2014) reported a mean straight line velocity (VSL) of 28.54µm/s for fresh semen of Sahiwal bulls. Though the trend showed higher VSL with advancement of age and SC, no significance difference was observed for age and SC variation in their study. Keshava (1996) also observed similar trends in Karan Freis cross breed bulls. The overall mean VSL of frozen semen reported by Ulfina *et al.* (2014) is 28.75µm/s at 0 hr after freezing and 30.24µm/s at 24 hr after freezing. Keshava (1996) reported nearly a similar result of 25.95µm/s for Karan Freis cross breed bulls. But Lenz *et al*. (2010) reported a little beat higher mean VSL (42.7 and 41.0µm/s by using 2-cell leja slide and 4-cell leja slide) respectively.

Table 1: Curvilinear velocity results of bovine semen obtained by CASA

Parameter	value	breed	Author(s)	Remark
	107.94µm/s			YA
	104.88µm/s			OA
VCL For Fresh semen	112.26µm/s			SSC

	110.01µm/s			LSC
VCL For Frozen semen (0hr)	94.45µm/s	Sahiwal	Ulfina *et al.* (2014)	YA
	90.72µm/s			OA
VCL For Frozen semen (24hr)	94.90µm/s			YA
	97.06µm/s			OA
VCL for fresh semen	87.10µm/s	Karan Freis	Keshava (1996)	
VCL for Frozen semen	92.0 µm/s		Lenz *et al*. (2010)	2-cell
	89.2µm/s			4-cell

YA=younger aged bulls, OA= older aged bulls, SSC= Smaller scrotal circumference LS= Larger scrotal circumference, 2-cell = by using 2-cell leja slide, 4-cell =by using 4-cell leja slide

The overall mean value of VAP reported by Ulfina *et al.* (2014) is 61.62µm/s for fresh semen of Sahiwal breed bulls. Similarly; a mean value of 62.28µm/s for fresh spermatozoa of the same breed and a slightly lower (50.22 µm/s) mean value for Karan Freis cross breed bulls were reported by Keshava (1996).Ulfina *et al.* (2014) reported a mean value of 58.29µm/s at 0 hrafter freezing and 56.58µm/s at 24 hr post freezing of Sahiwal breed bulls. Keshava (1996) reported a mean value of 52.58µm/s for Karan Freis breed bulls frozen spermatozoa. Similarly Lenz *et al*. (2010) also reported a mean of 50.3 and 48.3µm/s by using 2-cell leja slide and 4-cell leja slide respectively.

As it was reported by Christensen *et al.* (2005) linearity of spermatozoa has strong correlation with non-return rate to estrus. In agreement to this finding, Farrell *et al.* (1998) also reported high significant correlation (0.99) between bull fertility and CASA motility parameters evaluated for 59 day non-return rate to first service. A mean linearity of 27.78% for younger and 37.04% for older Sahiwalbulls fresh spermatozoa was reported by Ulfina *et al*. (2014). The authors also reported a mean linearity of 33.74% at 0 hrafter freezing and 34.82% after 24hr of freezing for Sahiwal bulls. The study also reported significantly higher percent linearity of frozen spermatozoa for older age group and for larger SC. Keshava (1996) also reported a similar result (34.48%) for Karan Freis cross breed bulls. Lenz *et al*. (2010) reported a slightly higher linearity percentage (48.9 and 48.6%) by using 2-cell leja slide and 4-cell leja slide respectively.

Mean values of 5.68µm for amplitude of lateral head displacement (ALH) at 0 hr after freezing and 5.88µm at 24 hr after freezing to Sahiwal bull spermatozoa were reported by Ulfina *et al.* (2014). Budworth *et al.* (1988), Farrell *et al.* (1998), Hoflack *et al.* (2007) and

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Amanda (2011) reported amplitude of lateral head displacement (ALH) value 3.2, 5, 4.83 and 8.45µm respectively for cryo-preserved spermatozoa. Lenz *et al*. (2010) also reported mean value of 5.4µm amplitude of lateral head displacement by using both type of slides (2-cell leja slide and 4-cell leja slide).

Budworth *et al.* (1988), Farrell *et al.* (1998), Hoflack *et al*. (2007) and Amanda (2011) reported a beat cross frequency (BCF) value of 15.9, 15, 37.4 and 30.99Hz respectively for cryo-preserved spermatozoa. Lenz *et al*. (2010) also reported a beat cross frequency (BCF) value of 24.0 and 23.8Hz by using 2-cell leja slide and 4-cell leja slide respectively.

Factors affecting CASA results

Numerous factors like type of chamber used for analysis, the temperature at which semen is analyzed, the concentration of spermatozoa to be analyzed, the type of extender in which semen is diluted, percent motility and digitization threshold can significantly affect CASA results (Davis and Katz, 1993).

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Specimen concentration: The inability of the CASA instrument to obtain accurate counts and percent motilities when the concentrations of the specimen is greater than 50 million per ml or less than 20 million per ml or when the specimen is laden with debris are fundamental limitations of the technology (Davis and Katz, 1993). This idea is also supported by Contri *et al*. (2010) that stats at low semen concentrations (less than 20 million per ml) an overestimation of the concentration and thus underestimation of the percentage of motile cells can occur due to the acquisition of non-spermatic particles (debris). On the other hand, at a higher concentration (above 50 million per ml) a large proportion of the fast moving cells will be excluded from analysis because of spermatozoa exiting the analysis area and cell collisions leading to underestimation of the motility percentage. For this reason laboratories have to be required either concentrate or dilute specimens which in turn severely limit the routine clinical application of the technology. Accuracy and precision of CON, MOT and kinematic variables are the most potentially affected parameters due to specimen concentration while analysis is done by CASA (Davis and Katz, 1993).

Percent motility: several earlier studies have reported inaccurate CASA results for sperm motility (Gill *et al*., 1988; Neuwinger *et al.,* 1990b; Davis and Katz, 1992). The inaccuracies

of CASA for percent motility were due to the error for overall sperm count and due to the bias to classify motile and immotile cells. In addition to that, the definition of motile sperm for visual and CASA approaches is not the same. In visual analysis, a spermatozoon is considered motile if its flagellum is twitching, even though it may have no forward progression. In CASA a spermatozoon must achieve a minimum VSL to be motile like 4.4µm/s which is set by Sundararaman *et al.* (2012). Hence, by definition, CASA measures will usually be lower than visual estimates, no matter how carefully the latter are done (Davis and Katz, 1993).

Specimen chamber: the type of specimen chamber used for analysis can affect the movement of sperm, the accuracy of the cell count and therefore the percentage of motile spermatozoa (Massányi *et al*., 2008). In agreement to this idea; as it is described above in the motility attribute Lenz *et al*. (2010) also reported a difference in values of different parameters for two types of specimen chamber (2-cell and 4-cell leja slide).

Temperature: the temperature at which semen is analyzed is also an important factor that can affect CASA results. Movement of sperm, particularly on MOT and VCL are the two most potentially affected parameters by the temperature. A decrease in the motility parameters (percentage of motile spermatozoa and track speed) was demonstrated by Vincent *et al.* (2012) when spermatozoa are not analyzed at a temperature of 37°C.

Sampling condition: it is also a source of error when acquiring data with CASA. Computer and video camera equipment are continuously evolving in the semen evaluation and different CASA systems use various models of video camera which could be the potential source for the result variation. Most of the CASA systems allow 30 Hz or 60 Hz as a frame rate to analyze sperm tracks and speed. Studies have shown the importance of the frame rate for reliability of the analysis (Brito, 2010; Castellini *et al*., 2011). It is generally accepted that a higher frame rate is required to render an evaluation closer to the real path for a fast non-linear sperm cell. Accuracy and precision of all measurements, especially VCL are the parameters which can be affected by frame rate (Davis and Katz, 1993).

Type of extender: the type of extender in which semen is diluted is another aspect that should be taken into consideration when evaluating spermatozoa with CASA; hence it affects many of the kinematic parameters. Some extenders contain debris of size similar to a sperm head,

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causing CASA software to include them in the analysis. Egg-yolk and milk based diluents are examples of extenders containing such particles. In addition, when observing semen diluted with milk extender, the globular lipids mask the spermatozoa thus rendering CASA analysis impossible (Davis and Katz, 1993).

Digitization threshold: For some years after the development of CASA technology; digitization threshold were been one of the factors affecting CASA result. Number of earlier studies have shown as image digitization threshold can significantly affect CASA results (Blach *et al.,* 1989; Toth *et al.,* 1991; Bendvold and Aenesen, 1990; Davis *and Katz,* 1992). Accuracy of sperm recognition and tracking are the two parameters those mainly affected by digitization threshold (Davis and Katz, 1993).

Table 2: Other possible factors that affect CASA results

Factor	Effect
Microscope optics and illumination methods	Accuracy of image digitization, target recognition and tracking
Laboratory supplies	Can be cytotoxic
Drop-to-drop variability	Accuracy and precision of all measures
Physiological state of the sperm	Rapidly swimming or capacitated sperm require a significantly higher video sampling rate to obtain accurate results
Number of frames analyzed	Accuracy and precision of all measurements
Number of sperm analyzed	Accuracy and precision of all measurements
Computational algorisms	Accuracy of VAP, ALH and BCF
Statistical methods	Accuracy and precision of all measurements

Adapted from Davis and Katz (1993)

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Flow cytometric assessment of sperm

Flow cytometry is used to analyze a variety of structural and functional characteristics of spermatozoa those can be detected by a fluorochromes or fluorescently labeled compounds through relative fluorescent intensity (Lindsay *et al.,* 2005). Sperm cell characteristics like plasma membrane integrity, chromatin structure, mitochondrial activity/mitochondrial membrane potential/, acrosome integrity, changes in the sperm surface induced by sperm capacitation and certain forms of morphological abnormalities present in a sperm sample are some of the structural and functional characteristics of spermatozoa detected by flow cytometry (Bochenek *et al*., 2001).

Fluorescently labeled sperm cells travel individually at high speed through a flow cytometer and illuminated by one or more lasers and this causes light scattering and fluorescence excitation of markers located on specific parts of the sperm, which is then picked up by photo detectors and sent to a computer program for presenting the information in the form of relative fluorescent intensity units, which are typically displayed as either scatter plots or histograms (Martinez-Pastor *et al*., 2010).

Key features of flow cytometry are the acquisition and analysis of thousands of cells within seconds and the multiparametric potential of the technology. Large numbers of spermatozoa can be analyzed in a very short period of time, generally at a rate of 8000–20,000 s^{-1}. A total of 10,000 spermatozoa are generally analyzed, which is substantially more than the total of 200 cells generally observed by microscopic analysis. This makes the flow cytometer a very sensitive method for the detection of subtle differences among spermatozoa that may not be apparent using other techniques (Lindsay *et al.,* 2005). Flow cytometry also has the capacity to detect more than one sperm attributes at a time. This feature has an added benefit for semen analysis, as few single sperm parameters show significant correlation with fertility in vivo for semen within the acceptable range of normality (Larsson and Rodrı´guez-Martı´nez, 2000); the more sperm parameters that can be tested, the more accurate the fertility prediction becomes (Amman. and Hammerstedt,1993).

The most modern cytometers are routinely equipped with three lasers and at least ten photomultiplier tubes allowing cell labeling with several probes at the same time thus enabling analysis of numerous parameters simultaneously. In the last few years, the multiparametric aspect of flow cytometry allowed this technology to become a popular tool to evaluate sperm attributes (Gillan *et al*., 2005; Martínez-Pastor *et al*., 2010; Hossain *et al*., 2011).

The presence of non-sperm events in the sample such as bacteria, blood cells, immature forms of spermatogenic cells, tissue, and extender contaminants in frozen-thawed semen (egg yolk particles) are the main concerns with flow cytometry sperm analysis. During the data analysis, these non-sperm events can be taken into account and most of the time can be eliminated from evaluation by gating of scatter diagram/Histogram (Chelsey and Peter, 2011).

Sperm attributes analyzed by flow cytometery

Viability/Mortality: Fluorescent staining of spermatozoa to determine viability can be approached either by fluorochromes used to indicate viable cells or by those used to indicate non-viable cells (Lindsay *et al.,* 2005). Fluorescein diacetate (FDA); 6-carboxyfluorescein diacetate (CFDA) or later, 6-carboxymethylfluorescein diacetate (CMFDA) and calcein acetomethyl ester (CAM) which tends to be more stable than the original FDA were used to assess viability of spermatozoa (Resli *et al.*, 1983; Garner *et al.,* 1986; Donoghue *et al.,* 1995). FDA, CFDA, CMFDA and CAM enter spermatozoa via the membrane and are converted by esterases in viable cells to a non-permeate fluorescent compound that retained in the cytoplasm (Lindsay *et al.,* 2005). More recently, membrane-permeate DNA fluorochromes, such as SYBR-14, which label viable cells with functional ion pumps, have become popular (Garner *et al.,* 1994). Sperm viability assessments using nucleic acid stains are considered to be less variable than enzyme-based stains and sperm DNA is believed to be a more appropriate cellular target due to its stainability and staining uniformity (Garner *et al*., 1996).

Non-viable cells can also be determined using membrane-impermeable nucleic acid stains which positively identify dead spermatozoa by penetrating cells with damaged membranes. An intact plasma membrane prevents these products from entering the spermatozoa and staining the nucleus. propidiumiodode (PI) is one of the fluorescent probe that binds to DNA and can identify non-viable cells. Live cells having an intact plasma membrane prevent PI from entering the cell, while cells with a damaged plasma membrane permit PI to enter the cell and subsequently PI binds to DNA and causes the cells to fluorescence red (Vincent *et al.,* 2012).

Combination of fluorochromes like CFDA along with PI, and SYBR-14 with PI are important to identify cell populations based on their viability. CFDA along with PI identifies three populations of cells: live, which are green; dead, which are red; and a third population staining in-between the two colors representing dying spermatozoa (Lindsay *et al.,* 2005). Almid and Johnson (1988) found this combination useful for monitoring membrane damage in frozen-thawed boar spermatozoa during evaluation of various freezing protocols. Harrison and Vickers (1990) also used this combination with a fluorescent microscope and found it to be an effective indicator of the viability of fresh, incubated or cold-shocked boar and ram

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spermatozoa. Garner *et al.* (1986) used this combination to stain spermatozoa from a number of species, but at that time they could not find a relationship between bull sperm viability detected by CFDA/PI and fertility.

As it was described in different studies (Partyka *et al*., 2010; Oldenhof *et al*., 2011; Vincent *et al*., 2012) combination of PI and membrane-permeate DNA stain (SYBR-14) sold commercially as LIVE/DEAD® Sperm Viability kit (Molecular Probes Inc., OR, USA) is an effective tool for assessing the viability of fresh as well as cryopreserved sperm in bovine semen. With these biomarkers, the DNA(nuclei) of live spermatozoa display green fluorescence because of integration of SYBR-14 and dead/dying cells with compromised membrane integrity stain orange(red) as a result of passive PI uptake through damaged plasma membrane and replacing the green fluorescence which were been stained before with SYBR-14 (Lindsay *et al.,* 2005; Vincent *et al*., 2012). As additional importance both of these probes (PI and SYBR-14) can be excited by 488nm laser (Vincent *et al*., 2012).

Acrosome integrity: The integrity of the acrosome is very closely associated with sperm viability because damage to the plasma membrane can trigger a disintegration of the acrosome. Plant lectins *Pivum sativum (pea) and Arachis hypogaea* (peanut) recognizing acrosomal ligands are used to study the integrity of the acrosomal membrane with flow cytometry (Anzar *et al*., 2011). *Pivum sativum (pea)* agglutinin binds to α-mannose and α-galactose moieties of the acrosomal matrix. As *Pivum sativum* agglutinin cannot penetrate the intact acrosomal membrane; only spermatozoa with damaged acrosome are stained (Nagy *et al*., 2003). However, it has been shown that *Pivum sativum* agglutinin has an affinity for egg yolk and non-specific binding sites on the sperm cell surface (Lybaert *et al*., 2009). This aspect could become a problem when analyzing semen frozen in egg yolk-based extender and result in misinterpretation of the acrosomal status of sperm. *Arachis hypogaea* (peanut) agglutinin binds to β-galactose moieties of the outer acrosome membrane and is the most popular lectin used to study the integrity of the acrosomal membrane with flow cytometry (Carvalho *et al*., 2010; Anzar *et al*., 2011; Yi *et al*., 2012). Spermatozoa with reacted, damaged, or abnormally formed acrosomes acquire green fluorescence after PNA labeling, while intact, normal acrosomes have no fluorescence (Nagy *et al.,* 2003). In addition, *Arachis hypogaea* (peanut) agglutinin seems the most reliable probe to identify spermatozoa with a

damaged acrosome as it displays less non-specific binding to other areas of spermatozoa. *Pivum sativum* agglutinin and *Arachis hypogaea* agglutinin are usually labeled with FITC fluorochromes, allowing them to be used by all cytometers (Lindsay *et al.,* 2005).

Mitochondrial activity: Mitochondria are very important organelles involved primarily in the generation of the energetic substrates for the motility of the sperm cell. Different probes like Rhodamine-123 (R-123), Mitotracker® (MITO) and JC-1 (5,5',6,6'-tetrachloro- 1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide) are used to monitor mitochondrial activity with the help of flow cytometer (Lindsay *et al.,* 2005).

Rhodamine-123 and Mitotracker® are transported into actively respiring mitochondria and their accumulation in the mitochondria causes them to fluoresce green. All functioning mitochondria stain green with R-123 and MITO and consequently no distinction can be made between spermatozoa exhibiting different respiratory rates (Hallap *et al*., 2005). R-123 is not suitable for use in experiments in which the spermatozoa are treated with aldehyde fixatives, since it will be washed out from the cell when the membrane potential is lost and this characteristic limits its use when quantification is needed or when fixation of spermatozoa is required. Whereas, the MITO probes accumulate, stain and retained in active mitochondria during the fixation process and has the advantage of availability in different ranges of excitation and emission fluorescence (Garner *et al*., 1997; Hallap *et al*., 2005; Sousa *et al*., 2011).

The mitochondrial stain 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) is a lipophilic cationic fluorescent carbocyanine dye that is internalized by all functioning mitochondria (Lindsay *et al.,* 2005; Gillan *et al*., 2005; Guthrie and Welch, 2008). It permits a distinction to be made between spermatozoa with poorly and highly functional mitochondria (Garner *et al*., 1997). In spermatozoa with mitochondria having a high membrane potential, JC-1 enters the mitochondrial matrix where it accumulates and forms J-aggregates and become fluorescent red. In spermatozoa having mitochondria with low membrane potential, JC-1 cannot accumulate within the mitochondria and remains in the cytoplasm with a green fluorescent monomeric form (Vincent *et al*., 2012). JC-1 has the advantage to quantify the mitochondrial burst of the cell compared to Rhodamine -123 and Mitotracker. A disadvantage of JC-1 probe is its dual fluorescence emission that limits its

combination with other probes emitting in the green and red fluorescence (Lindsay *et al.,* 2005).

Sperm DNA Integrity: Integrity of DNA packaging within sperm chromosomes has a biological correlate with fertility and is the most important determinant factor for the embryo development and offspring production (Erenpreiss *et al.,* 2006). As it was described by Genesca *et al.* (1992) DNA fragmentation is characterized by both single and double DNA strand breaks.

DNA damage (abnormal chromatin structure) may arise from different processes: some of the possible processes for development of abnormal chromatin structure are deficiencies in recombination during spermatogenesis, abnormal spermatid maturation caused by protamination disturbances and endogenous mechanisms such as abortive apoptosis, and oxidative stress (Erenpreiss *et al.,* 2006). Loss of DNA integrity does not always impair fertilization, but compromises sustainable embryo development which can predispose to embryo losses and abortion (Watson *et al.,* 2000; Samplaski *et al.,* 2010).

Though exactly what is measured with each assay differs; evaluation of sperm DNA integrity can be achieved by variety of tests covering different aspects of the DNA damage. Unfortunately, most of the available techniques provide limited information regarding the nature of the DNA lesions and do not allow to highlight the exact pathogenesis of disrupted sperm DNA (Bungum, 2012; Agarwal *et al.,* 2004). Acridine orange, aniline blue, chromomycin α and toluidine blue are some of the less expensive methods used to assess the sperm chromatin structure using chromatin structural probes or dyes (Evenson *et al.,* 2006). Though these are the less expensive methods, TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP (2´-deoxyuridine, 5´-triphosphate) nick end labeling) assay and sperm chromatin structure assay (SCSA) are the most currently used tests of sperm DNA fragmentation under the application of flow cytometer (Natali and Turek, 2011).

Sperm Chromatin Structure Assay is a measure for level of DNA fragmentation in the sperm based on the assumption that a structurally abnormal sperm chromatin and sperm with immature chromatin shows a higher susceptibility to acid denaturation due to less chromatin condensation (Chohan *et al.,* 2006; Evenson *et al.,* 2002). As it was described by Lewis and

Aitken (2005) the spermatozoal chromatin is much more compact when compared to somatic and spermatogenic cell types (spermatognia, spermatocytes and spermatids). The SCSA method utilizes the metachromatic properties of acridine orange (AO) that can fluoresce in the green band when intercalates into the intact double-stranded DNA helix and the red band when associated with single strand denatured DNA (Bochenek *et al.,* 2001). When performing SCSA, the cells pass through a laser beam set with a flow cytometer and the light from the beam causes the dye to emit fluorescent light of red and green. Green fluorescing sperm have very low (or no) level of fragmented DNA and red fluorescing sperm have moderate to high levels of fragmented DNA (Bochenek *et al.,* 2001). DNA fragmentation level greater than 30% of the total sperm cells is likely to have significant reduction in fertility potential and pregnancies (Brahem *et al*., 2011).

Table 3: DNA fragmentation index (DFI)

DNA Fragmentation index (DFI)	Result
< 15%	Excellent DNA
15-24%	Good DNA
25-29%	Fair DNA
≥30%	Poor DNA

Source: (Brahem *et al*., 2011)

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Sperm chromatin structure could be affected and high level of DNA fragmentation can be recorded by different factors like length of sexual abstinence, age, testicular cancer, trauma and exposures to prolonged heat, high levels of air pollution, chemicals and/or radiation (Brahem *et al*., 2011). Therefore, while performing sperm chromatin structure assay taking into consideration for such conditions and or events is important.

DNA fragmentation in spermatozoa can also be assessed using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, which identifies DNA strand breaks by labeling free 3'-OH termini with modified nucleotides (Duran *et al.,* 2002). Transferase enzyme incorporates and fluorescent or modifies nucleotides at the sites of DNA breakage and labeled cells can then be analyzed by flow cytometry (Waterhouse *et al*., 2006). Duran *et al.* (2002) found that human semen samples with greater than 12% of the

spermatozoa containing DNA fragmentation did not result in pregnancy and Benchaib *et al.* (2003) obtained no pregnancies if this value was greater than 20%.

Limitations of flow cytometry

Several factors restrict flow cytometer usage and influence the results of semen quality parameters obtained by it: price of the instrument, number of laser and photomultiplier tubes for its multiparametric analysis, the need for type of analyses to be performed, sophistication of some of its software, unique characteristics of spermatozoa and the type of extender in which semen is diluted are some of the factors restricting and/or influencing the cytometer usage for analysis of sperm cells (Vincent *et al.,* 2012).

Multiparametric analysis is usually obtained with instruments containing more than one laser and many photomultiplier tubes which substantially increase the price of the equipment and in turn restricting the choice of flow cytometer for analysis of semen. The type of analyses to be performed is also a factor that will determine the choice of the flow cytometer; depending on the objectives of the breeding center and the experimental design, the combination of lasers (number and wavelength) and the number of photomultiplier tubes included in the instrument must be taken into account. An instrument with only one laser and three photomultiplier tubes allows detection of a maximum of three parameters on each cell; whereas four and more multiparametric analyses usually require an instrument having at least two lasers and four photomultiplier tubes. The software operating the flow cytometer is another important aspect in the choice of the instrument. Most software products available are fairly easy and straightforward to operate for a novice user in flow cytometry. However, some of its software requires certain knowledge of flow cytometry concepts, making the instrument more difficult to operate. The unique characteristics of spermatozoa must be considered when selecting an instrument; the paddle shape of the head and presence of the flagellum make spermatozoa very different in size and cellular complexity compared to most cells studied by flow cytometry. Indeed, the majority of cells studied with this technology have round shape; and passage of them in front of the laser leads to a neat forward scatter vs. side scatter plot. However, when a sperm cell hits the laser, it could be on the thick or on the thin side of the head. This unique feature of sperm cells will lead to a scatter plot of different size/complexity. The extender in which semen is diluted is also a very important aspect; as different types of

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extenders are used to dilute semen, some contain particles of a similar size to spermatozoa. This aspect of particle contamination of the target population is a concern when considering a cytometer for multiparametric analyses of semen.

Relative importance of CASA and Flow cytometer

As compared to conventional subjective evaluation of semen which is relatively inaccurate, imprecise, time consuming and dependent on the level of training and skills of the investigator (Christensen *et al*., 2005); computer-assisted sperm analysis is a useful tool for identifying differences in sperm parameters related to motility, velocity and morphology which have significant contribution for predicting fertility of an individual bull. The kinematic values, the width of the sperm head's trajectory and frequency of the change in direction of the sperm head determined for each spermatozoon provide qualitative and quantitative assessment of sperms (Patel and Dhami, 2013).

Flow cytometry is fast, accurate, highly repeatable and can analyze significantly more sperm per sample (up to 10,000) than standard semen analysis; even to that of CASA (Christensen *et al.,* 2005). In addition to the speed, repeatability and accuracy; flow cytometry allows close examination of numerous structural and functional characteristics of spermatozoa: sperm viability/membrane integrity (Christensen *et al.,* 2005), mitochondrial function and membrane potential (Garner *et al.,* 1997) chromatin structure (Bochenek *et al.,* 2001), and acrosomal status (Nagy *et al.,* 2003). Flow cytometry is also used for the simultaneous evaluation of DNA integrity, oxidative status, membrane fluidity and permeability, lipid peroxidation, and tyrosine phosphorylation of sperm proteins with the help of different probes (Peña, 2007; Ortega-Ferrusola *et al*., 2009a). And by using combination of probes "alien" particles can also be easily gated out from the analysis. For example out of multiple dot plots that can be developed in the sample while undertaking flow cytometer analysis, dot plots combining Hoechst 33342 with propidium iodide or Ethidium homodimer can be used to determine the percentage of live and dead spermatozoa in that sample if these probes are in use for the analysis.

A multiparametric approach to semen analysis using CASA, flow cytometry and preferably in combination is the fundamental advanced technique to screen the sub-fertile bulls by undertaking objective, repeatable, accurate and rapid tests. Semen quality assessment by using

Flow cytometry and CASA not only save considerable amounts of money for the producers as well as for the country but also give relatively best quality semen that was assessed in different ways of parameters. For this reason it is exceedingly preferable that semen laboratories shall be use multiparametric approaches of CASA and flow cytometry simultaneously that can right-handily make possible the semen production centers to produce high quality semen than the one assessed by one of the technique alone. However, if prioritization is the driving force for different reasons (like economic reasons to afford both) flow cytometery is relatively good technology for its structural and functional semen analysis even if it is coasty as compared to that of CASA.

CONCLUSION AND RECOMMENDATIONS

For more than half a century, scientists had struggled to develop laboratory assays that precisely estimate and/or predict the fertilizing capacity of semen. An important issue for a laboratory analysis to be useful is that, it must be objective, repeatable, accurate and as far as possible, rapid. Semen analysis could be either conventional or advanced. Conventional methods used in sperm quality assessment are unsatisfactory to correctly predict sperm fertility potential and do not provide sufficient information for diagnosing and overcome some clinical infertility situations. Advanced evaluation tests of sperm for its morpho-functional status have the increased potential to deliver relevant information and allow an increased efficiency in the identification of infertile individuals. The sperm motility and kinematic parameters measured by CASA are repeatable, reliable and objective in their nature and can provide reflections up on semen quality. Similarly, Flowcytometry is also another advanced method used to assess the semen quality and in turn fertility potential of the bull in a fast and objective manner. However, as there is no absolutely beneficial technology, these systems also have their own drawbacks; for instance in case of CASA; results may vary due to type of chamber used, the temperature at which semen is analyzed, the concentration of spermatozoa and the type of extender in which semen is diluted. Similarly, in Flow cytometery; factors like price of the instrument, number of laser and photomultiplier tubes and sophistication of some of its software are some of the factors that restrict the extensive cytometer usage for analysis of sperm cells. Based on this conclusion, we would like to

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recommend that screening the sub-fertile bulls by under taking objective, repeatable, accurate and rapid tests for their semen quality assessment using Flow cytometry and CASA potentially save considerable amounts of money for the producers as well as for the country; especially if this condition is done in the early age of the bull and in semen production center. And for this reason it is exceedingly preferable that semen laboratories shall be use multiparametric approaches of CASA, flow cytometry and preferably in combination which can right-handily make possible the semen production centers to produce high quality semen.

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ANNEXES

Annex 1: Standard procedure for CASA

Factor	Recommendation
Specimen chamber (type, depth and method of sampling)	Reusable chambers should be periodically checked for accuracy and discarded after their recommended life time has been exceeded.
Temperature of analysis	The best temperature is probably the normal physiological temperature for the species.
Specimen concentration	Specimen must be diluted to < $50*10^6$ sperm per ml to obtain accurate measures for CON and MOT.
Laboratory supplies	Each lot that comes into contact with sperm must be screened for cytotoxicity
Video framing rate	Fast swimming sperm should be evaluated at a faster video framing rate(60fps rather than 30fps). Failure to use the appropriate video framing rate will result in significant errors in VCL, BCF, ALH, LIN, WOB and STR.
Number of frames analyzed	. In cell track CON and MOT are only accurate when no more than five video frames are analyzed at 30 fps
Number of sperm analyzed	At least 200 motile sperm should be analyzed to obtain accurate estimates of population means, and at least 300 motile sperm to obtain accurate estimates of population distributions.
Number of field to analysis	A pattern of chamber sampling should be developed that minimizes the between field variation.
Number of drops to analyze	At least two drops should be analyzed and their difference computed. If the difference exceeds the value defined for quality control purposes, then a third drop should be analyzed. The median of the three drops should be used.

Source: (Davis and Katz, 1993).

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Annex 2: Analysis set-up used to evaluate bull spermatozoa

Variables	Settings
Frame rate (Hz)	60
Frames acquired	30
Minimum contrast	40
Minimum cell size (pixels)	8
Threshold straightness (%)	64
Medium VAP cut-off (µm/s)	80
Low VAP cut-off (µm/s)	15
Low VSL cut-off (µm/s)	4.4
Non-motile head intensity	80
Static size limit – minimum	0.38
Static size limit – maximum	1.49
Static intensity limit – minimum	0.42
Static intensity limit – maximum	1.35
Static elongation limit – minimum	12
Static elongation limit – maximum	81
Magnification	1.89
Camera frequency (Hz)	60
Chamber depth (µm)	10
Motile position (µm)	16.3
Static position (µm)	16.3

Source: (Sundararaman *et al.,* 2012)

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