

## Guidelines on the application of CASA technology in the analysis of spermatozoa



### ESHRE Andrology Special Interest Group\*

#### Introduction

The recommendations contained in this document represent the consensus position of the ~35 participants attending a computer-assisted semen analysis (CASA) Workshop\*\* held at San Miniato (Pisa), Italy on 3–6 April, 1997. In the Workshop, the theory and principles of digital image analysis of static and moving spermatozoa were presented, and the practical applications of CASA technology in research and clinical settings, both now and in the future, were discussed.

We urge that the recommendations be considered carefully by CASA users and manufacturers alike. The further development of CASA requires that a cooperative approach be taken by all parties.

It should be noted that all participants in the Workshop agreed strongly that CASA manufacturers must provide more comprehensive training and support programmes for CASA users. While this point has been included in the recommendations below, it must be emphasized that we consider that improved training in both the principles of CASA technology and the use of CASA instruments is necessary before further, meaningful progress can be made in its clinical application.

Several recommendations reiterate verbatim ones contained in the ESHRE Andrology Special Interest Group's previous consensus workshop (ESHRE Andrology Special Interest Group, 1996).

#### General recommendations for all mammalian species

1. Users must recognize that standardization and quality control (both internal and external) are paramount fundamental

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\*The CASA Workshop was organized by Domenico Canale (University of Pisa) on behalf of the ESHRE Andrology Special Interest Group (SIG). The Guidelines, based on discussions conducted at the conclusion of the Workshop, were drafted by Sharon Mortimer, David Mortimer and Lynn Fraser<sup>1</sup>, Coordinator of the SIG. All participants and members of the SIG had the opportunity to suggest modifications before the final document was drawn up. Professor R.G.Edwards, Editor of *Human Reproduction*, supports our recommendation that studies using CASA techniques should conform to these Guidelines.

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requirements for CASA, just as they are for any other diagnostic andrology laboratory procedure.

2. Manufacturers are urged to provide more extensive user-oriented training in the routine use of CASA instruments beyond simple installation and setup instructions. This training should include specific advice on handling of specimens and making preparations for analysis. We suggest that manufacturers should consider offering such training courses as satellites to national/international meetings.

3. As for all measurement systems, it is the responsibility of users to ensure that an appropriate number of cells is sampled in order to achieve the statistical requirements for a proper interpretation of the results obtained with CASA, including sensitivity and specificity. This is essential if CASA-derived information is to be used in decision-making in a clinical context.

4. As presented previously (ESHRE Andrology Special Interest Group, 1996), it was agreed that a primary factor affecting the performance of a CASA instrument is the technical competence of its user. It is mandatory that technicians are trained to understand the theory behind CASA, as well as the influence that the initial settings can have on the data produced (see Tables I and II; ESHRE Andrology Special Interest Group, 1996).

5. Quality control (QC) is extremely important, and each laboratory using CASA must have QC procedures. It has been suggested that for sperm concentration, CASA results should be compared directly with manual assessments using an improved Neubauer haemocytometer over a wide range of sperm concentrations. For sperm motility analysis, it has been suggested that prepared videotapes be used, as well as utilizing the 'playback' option of the CASA instrument (if it has one) to ensure that all motile spermatozoa are being identified using the current setup parameters. As a **minimum**, these QC procedures should be run when the machine is switched on for use and each time after the operating parameters have been changed. All CASA systems should be equipped with common video inputs and outputs that allow QC with videotapes (at 50 or 60 Hz sample rate) (Davis and Katz, 1993; Clements *et al.*, 1995).

6. In any manuscript or report, methods should be presented in sufficient detail to establish that accepted relevant CASA guidelines were followed. These include: image acquisition rate, track sampling time, smoothing algorithm employed, number of cells sampled, type of chamber including its depth, as well as instrument model and software version numbers, and microscope optics and magnification.

7. Chamber depth must be sufficient for unconstrained sperm

motion. This will vary depending on the species. In addition, chamber depth must match optics to achieve appropriate depth of focus (Makler, 1978).

8. In all CASA analyses, the user must verify that all motile spermatozoa in the field of view are being tracked.

### Basic instrumentation

9. When used for kinematic analysis of non-capacitated spermatozoa in seminal plasma, the magnification of the CASA instrument should be such that the majority of spermatozoa are tracked for 0.5 s before leaving the field of view. Hence, for human spermatozoa, the minimum field of view should be no less than  $200 \times 200 \mu\text{m}$ , allowing optimal tracking of spermatozoa with straight line velocity values up to  $100 \mu\text{m/s}$ .

10. For human spermatozoa, an objective with maximum  $\times 10$  magnification and suitable numerical aperture must be used so as to provide an adequate depth of focus.

11. For motility analysis, the digitizer should have maximum resolution consistent with standard video technology.

12. Reliable analysis of human sperm motility ideally requires a minimum acquisition frequency of 50 Hz (Mortimer *et al.*, 1988).

13. A minimum sampling time of 0.5 s is needed to acquire reliable kinematic values for a track. CASA system parameters should be set to achieve this whenever possible. If data from a sample include a large proportion of shorter tracks, then users must be aware that this may bias their data and prejudice results. If longer trajectories are used, it must be demonstrated that this does not affect the results by excluding fast spermatozoa that leave the field, by skewing the population by including additional fast cells that enter the field during a prolonged acquisition window, or by counting broken segments of individual tracks as multiple spermatozoa.

### Determination of sperm concentration

14. This must not be a primary reason for acquiring a CASA instrument.

15. If a user wishes to use a CASA instrument to determine sperm concentration then he/she must establish that the intended measurement procedure provides accurate results compared with established, reliable techniques (e.g. WHO haemocytometry method and perhaps flow cytometry; WHO, 1992).

16. It is considered that the current generation of CASA instruments does not provide accurate, reproducible values for sperm concentration unless the method can differentiate spermatozoa from other cells and debris by a specific staining method, e.g. fluorescent staining of DNA with quantitative determination of nucleus size (Zinaman *et al.*, 1996).

### Determination of the proportion of motile spermatozoa

17. This should not be considered a primary reason for acquiring a CASA instrument.

18. If used in the routine analysis of seminal spermatozoa, CASA should be used to determine the **concentration** of

progressively motile spermatozoa. CASA can determine this value accurately if care is taken with specimen preparation, instrument use and appropriate user-defined criteria (Mortimer *et al.*, 1995).

Current CASA instruments should not be used for the determination of the **proportion** of motile spermatozoa, since they cannot be relied upon to distinguish between debris and dead spermatozoa while tracking live spermatozoa at the same time.

### Determination of sperm movement

19. For the time being, it is agreed that: (i) semen samples with sperm concentrations higher than those recommended by the CASA instrument manufacturer must be diluted using cell-free autologous seminal plasma; (ii) all CASA analyses should be performed at body temperature ( $37^\circ\text{C}$  for human spermatozoa); (iii) a minimum chamber depth of  $10 \mu\text{m}$  must be used. Depths  $>20 \mu\text{m}$  are unlikely to be of any benefit; and (iv) at least 200 motile spermatozoa should be analysed per sample.

20. Studies should be undertaken to develop a standardized test of seminal sperm kinematics in artificial medium after separation from seminal plasma so that the concentration of motile spermatozoa can be adjusted to allow optimum performance of the CASA instrument. The medium used should be non-capacitating.

21. Because the derivation of the average path of uncapacitated spermatozoa is dependent upon both the image acquisition frequency and individual track geometry, CASA instrument manufacturers are strongly urged to develop software which allows appropriate smoothing of each sperm track individually (Davis *et al.*, 1992).

22. For seminal spermatozoa (either in seminal plasma or a standard test medium) population-averaged kinematics are considered to be of limited value. This is because values are often not normally distributed, making the mean an inappropriate estimate of the sample. Consequently: (i) median and range or centile values are considered to be statistically more meaningful than mean values; (ii) multiparametric kinematic definitions, which would allow the classification of individual spermatozoa into specific subpopulations which are correlated with relevant functional endpoints (e.g. 'good mucus penetrating' spermatozoa), should be made.

For critical analysis of data it is recommended that individual track values be saved in a database so that their distribution may be examined in order to select the most appropriate statistic for either describing the sample or comparing it with others (e.g. fertile versus infertile men or treated/exposed versus control/unexposed animals).

23. CASA manufacturers are strongly urged to develop more intelligent track reconstruction software. In particular, vector tracking to resolve confused tracks such as those between colliding spermatozoa or wrongly-combined leaving-the-field/entering-the-field composite tracks. This will reduce the concentration-dependency of CASA instruments to some extent, although probably not eliminate it.

### CASA analysis of hyperactivation

**24.** Time-averaged kinematic measures must be employed with extreme caution in the identification of hyperactivated spermatozoa. It is recommended that modern studies should focus on dimensionless, machine-independent measures of movement patterns (e.g. Davis and Siemers, 1995; Mortimer *et al.*, 1996).

**25.** Spermatozoa for hyperactivation studies should not be prepared from liquefied semen using techniques that have been demonstrated to have potentially deleterious effects on sperm function, e.g. initial centrifugation to separate spermatozoa from seminal plasma (e.g. Aitken and Clarkson, 1988; Mortimer, 1994).

**26.** An appropriately-constituted culture medium should be employed, i.e. one capable of supporting capacitation *in vitro*. However, the medium must contain at least 25 mM  $\text{HCO}_3^-$  and mM quantities of  $\text{Ca}^{2+}$  and glucose. Sufficient albumin, probably at least 10 mg/ml, must be incorporated to minimize the sticking-to-glass phenomenon.

It should be borne in mind that media containing iron (e.g. Ham's F-10) have been shown to promote the formation of reactive oxygen species (ROS) during extended culture of human spermatozoa (Gomez and Aitken, 1996).

**27.** All hyperactivation analyses must be performed at body temperature (i.e. 37°C for human spermatozoa).

**28.** Preparations of sufficient depth so as not to constrain hyperactivated movement must be used. For human spermatozoa, a minimum chamber depth of 30  $\mu\text{m}$  is essential. Although 50  $\mu\text{m}$  is probably ideal, it can only be employed if the optical system used is capable of resolving this depth of focus (Le Lannou *et al.*, 1992).

**29.** Definitions for hyperactivated motility must take into account the image acquisition frequency and should use kinematic criteria which have been validated for that CASA instrument (Mortimer and Swan, 1995; Morris *et al.*, 1996).

**30.** It is recommended that machine-independent (smoothing-independent) kinematic measures of sperm movement be developed: (i) to allow easier comparison of results between CASA instruments and between laboratories; (ii) to take into account the differences between regular, non-hyperactivated track patterns and the less regular, hyperactivated patterns.

### Morphology assessment by CASA

**31.** The current generation of CASA instruments is not capable of analysing human sperm morphology in a manner adequate for routine clinical applications. In particular, the inability to include assessment of the midpiece and tail regions (required by the WHO guidelines) is considered to be a major weakness. Consequently, the use of CASA instruments for the clinical assessment of human sperm morphology is not supported at this time (WHO, 1992).

**32.** Development of a standardized protocol for the preparation of human spermatozoa for morphological analysis (including both the making and staining of preparations) using CASA technology should be pursued as a matter of high priority

### Clinical application of CASA

**33.** In the context of male fertility diagnosis, CASA should not be used without first undertaking a proper clinical assessment of the patient (including a clinical history and physical examination).

**34.** For the present, CASA should not be undertaken in a clinical setting without first having constructed a basic semen profile according to recognized (WHO) guidelines.

**35.** Clinicians should understand that correct sample handling prior to analysis is critical for obtaining useful information. This is true for all semen assessments, not just CASA. Hence the length of abstinence (ideally in hours) and the ejaculation-to-analysis delay must be known accurately.

**36.** Studies are required, using the current generation of CASA systems, to define: (i) the limits of normal semen quality in fertile men; (ii) the relationships between CASA variables and the time to pregnancy; (iii) the relationships between CASA variables (especially hyperactivation) and IVF outcome. Recent relevant studies include Irvine *et al.* (1994) and Macleod and Irvine (1995).

### Applications of CASA in reproductive toxicology

**37.** For toxicological studies on epididymal or vasa deferentia spermatozoa, accepting that samples must be diluted, the user must demonstrate that: (i) the process of dilution does not damage the spermatozoa; (ii) the medium used supports consistently normal motility over the period of analysis; (iii) the sperm concentration has been adjusted and controlled to minimize artefacts induced by crowding.

**38.** Magnification should be adjusted according to sperm size and velocity (e.g.  $\times 4$  is appropriate for rat spermatozoa which are considerably larger and faster than human spermatozoa) and chamber depth should allow unimpeded motion (e.g.  $\geq 40 \mu\text{m}$  for rat spermatozoa).

**39.** Users must verify that instrument settings have been optimized to detect all motile spermatozoa.

**40.** Users must verify that the temperature at which images are captured is the body temperature for that species.

**41.** With rodent sperm preparations, CASA can be used to determine both percentage motile and percentage progressively motile. User-defined thresholds for progressiveness should be justified based upon distributions of these endpoints in control samples.

**42.** A permanent record of samples (e.g. video or digital recording) is recommended so that data can be verified at any time, and re-analysed as necessary when improvements in software become available. Fields should be recorded in a predetermined order so that the user does not bias the results by selecting or rejecting fields on the basis of their appearance.

**43.** New users should compare their sample preparation methods and velocity data with those in the literature to achieve minimum standards in line with current practice. Recent relevant consensus reports include Schrader *et al.* (1992) and Seed *et al.* (1996).

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