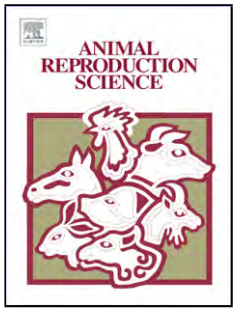

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Toward an integrative and predictive sperm quality analysis in *Bos taurus*

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Highlights

- The new multi-parametric method allows, for the first time, the assessment of membrane, acrosomal integrity and motility parameters in the same spermatozoa.
- The new method allows to observe live spermatozoa showing signs of capacitation such as hyperactivated motility and changes in acrosomal structure.
- Different patterns of motility were described for the different fluorescence sperm subpopulations.

ABSTRACT

There is a need to develop more integrative sperm quality analysis methods, enabling researchers to evaluate different parameters simultaneously cell by cell. In this work, we present a new multi-parametric fluorescent test able to discriminate different sperm subpopulations based on their labeling pattern and motility characteristics. Cryopreserved semen samples from 20 Holstein bulls were used in the study. Analyses

of sperm motility using computer-assisted sperm analysis (CASA-mot), membrane integrity by acridine orange-propidium iodide combination and multi-parametric by the ISAS®3Fun kit, were performed. The new method allows a clear discrimination of sperm subpopulations based on membrane and acrosomal integrity, motility and morphology. It was also possible to observe live spermatozoa showing signs of capacitation such as hyperactivated motility and changes in acrosomal structure. Sperm subpopulation with intact plasma membrane and acrosome showed a higher proportion of motile sperm than those with damaged acrosome or increased fluorescence intensity. Spermatozoa with intact plasmalemma and damaged acrosome were static or exhibit weak movement. Significant correlations among the different sperm quality parameters evaluated were also described. We concluded that the ISAS®3Fun is an integrated method that represents an advance in sperm quality analysis with the potential to improve fertility predictions.

Keywords: bull; ; ; ; , semen, sperm quality, CASA, multi-parametric

1. Introduction

Sperm evaluation techniques have greatly evolved in recent decades, especially with the incorporation of new methods of fluorescent staining, flow cytometry and computer-assisted sperm analysis (CASA) systems. Fluorescent markers and flow cytometry allow the assessment of numerous structural and functional characteristics of spermatozoa in large populations (Martinez-Pastor *et al.*, 2010; Robles and Martinez-Pastor, 2013). CASA systems allow studying the motility and morphometry of spermatozoa in a much more detailed and objective way (Verstegen *et al.*, 2002; Lu *et*

al., 2014; Yániz *et al.*, 2015b). All this makes it possible to analyze the type and extent of sperm damage more precisely.

In spite of these advances, the predictive capacity of the in vitro analysis on potential fertility of semen is still limited (Rodriguez-Martinez, 2003; Santolaria *et al.*, 2015), although it may be improved using combined statistical analyses of various sperm quality parameters (Sellem *et al.*, 2015; Utt, 2016). In consequence, the complexity of the sperm analysis has progressively been increased with the hope of improving fertility predictions (Fraser *et al.*, 2001; Yániz *et al.*, 2008a). However, some of these quality parameters are highly correlated, with merely increasing the number of analytical tests does not always improve the predictive ability of the spermiogram (Brito *et al.*, 2003; Utt, 2016).

A limitation of in vitro quality testing is that different parameters are usually assessed separately on the sample, losing the power of variables integration. There is a need to develop more integrative sperm quality analysis methods, enabling us to evaluate different parameters simultaneously cell by cell. With this aim, different fluorescent probes have been combined to obtain multi-parametric determinations of sperm quality. Fluorescent conjugates have been prepared to simultaneously assess sperm membrane and acrosome integrity (propidium iodide/FITIC-PSA or FITC-PNA; Peña *et al.*, 1999; Nagy *et al.*, 2003). Other combinations, such as IP/FITC-PSA/JC-1 (de Andrade *et al.*, 2007) or IP/bis-benzamide/Mitotracker Green-MF/SBTI (Bussalleu *et al.*, 2005), also allow to determine the mitochondrial activity. Despite achieving multi-parameter determinations, these treatments may cause alterations in spermatozoa, such as loss of motility. In this work, we present a new multi-parametric fluorescent test with the

potential for improving fertility predictions that is able to discriminate different sperm subpopulations based on their membrane and acrosome integrity, functionality and motility characteristics.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, all chemicals products were purchased from Sigma Chemical Co. (Madrid, Spain), and Milli-Q water (Millipore Ibérica S.A., Barcelona, Spain) was used for the solutions preparation.

2.2. Semen samples

Animal Care and Use Committee approval was not obtained for this study because no animals were used. Cryopreserved semen samples from 20 commercial Holstein bulls were used in the analyses. Straws with 0.25 mL of frozen semen were thawed for 1 min at 37 ºC in a water bath and processed for sperm quality assessment.

2.3. Sperm quality assessment

2.3.1. Assessment of sperm motility by computer-assisted sperm analysis (CASA-Mot).

Computer-assisted sperm analyzer (ISAS®, Version 1.1, PROISER, Valencia, Spain) was used to assess sperm motility (Palacín *et al.*, 2013). Two consecutive drops and at least 500 sperm cells were analyzed by CASA-Mot (Soler *et al.*, 2016) for each sample.

2.3.2. Evaluation of sperm plasmalemma

Sperm viability (membrane integrity, SV) was determined using acridine orange (AO) and propidium iodide (PI) (Yániz *et al.*, 2013c; b) using the DUO-VITAL kit (Halotech, Madrid, Spain). At least 200 sperm cells were examined per sample.

2.3.3. Multi-parametric assessment with the ISAS®3Fun kit.

Samples were labeled with the ISAS®3Fun kit (Proiser, Paterna, Spain) developed by the TECNOGAM research group. Briefly, 40 µl sample aliquot was pipetted into 0.6 ml Eppendorf centrifuge tubes, 4 µl of the fluorochrome combination provided by the kit was added and samples were incubated for 5 min at 37ºC in a water bath. Sample aliquots were directly placed in a prewarmed slide, covered, and assessed with fluorescence microscopy to evaluate the motility of fluorescent sperm subpopulations.

Digital images of the fluorescence-labelled sperm were obtained using an epifluorescence microscope (DM4500B Leica, Wetzlar, Germany) equipped with warmed stage and a triple-band bandpass filter (B/G/R triple-band filter cube, Leica, Wetzlar, Germany). A JenOptik ProgRes CF CCD (JenOptik AG, Jena, Germany) coupled with Jenoptik Progress Capture Pro image acquisition software was used for the evaluation of motility using time-lapse. Detection of the different fluorescent subpopulations and evaluation of their motility characteristics was performed with the ISAS® software (ISAS®, Version 1.1, PROISER, Valencia, Spain). The motility variables measured included the sperm cell motility percentage (MS, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), average path velocity (VAP, µm/s), sperm linearity (LIN, as a measure of a curvilinear path, VSL/VCL), straightness (STR, as the linearity of the average path, VSL/VAP), wobble (WOB, oscillation measure of the

actual path about the average path, VAP/VCL), and amplitude of lateral sperm head displacement (ALH, μm).

For a more detailed study of the morphology of sperm fluorescent subpopulations, digital images were obtained from the samples immobilized with formaldehyde, using the same microscope as described above equipped with a 63X plan apochromatic objective, and photographed with a Canon Eos 400D digital camera (Canon Inc., Tokyo, Japan). The camera was controlled by a computer by using DSLR Remote Pro software (Breeze Systems, Camberley, UK).

2.4. Statistical analyses

Statistical analyses were performed using the SPSS package, version 15.0 (SPSS Inc., Chicago, IL, USA). Normality distributions and variance homogeneity of the median value score for each set were checked by the Kolmogorov–Smirnov and Levene tests, respectively. As data of sperm motility in the fluorescent stained samples were non-normally distributed, the Kruskal–Wallis test was used for comparison of motility, followed by the Mann–Whitney a posteriori test. Spearman's correlation coefficient was used to assess the correlations between the different sperm quality parameters.

3. Results

The new method used allows us to distinguish intact or damaged plasma membranes and acrosome (Fig. 1). The basic components of the sperm head, nucleus and acrosome were perfectly defined. The flagellum was also visible in some spermatozoa with increased fluorescence.

The new procedure can differentiate different fluorescent sperm subpopulations based on cellular functional state (Figs 2, 3). It is possible to observe the evolution from a living sperm with intact acrosome to another with total acrosomal loss (Fig 2). Sperm at intermediate stages showed increased acrosome and flagellar fluorescence intensity (Fig. 2b, 2c), and progressive fading of the acrosome, maintaining high flagellar fluorescence intensity (from Fig. 2d to 2g). It is also possible to follow the evolution from a live sperm with intact structures to a dead sperm without acrosome (Fig 3). At intermediate stages, a progressive loss of acrosome integrity may be appreciated (Fig. 3c to 3e).

The study of sperm motility of labelled spermatozoa is also possible using the new fluorochrome combination (Fig. 4). The fluorochromes included in the kit were selected on the basis of having a low impact on sperm motility, together with providing good quality images. The effect of adding fluorochromes on sperm motility was checked using CASA-mot. A decrease of 15 % in total sperm motility was observed after adding fluorochromes, but it was well maintained afterward and the other kinematic variables were not affected. We assumed that this effect is not relevant since affects equally all individuals, whereby the comparison between different individuals is acceptable.

All spermatozoa with damaged plasma membrane were static. Table 1 shows the motility characteristics of the three sperm functional classes. Sperm subpopulation with intact plasma membrane and acrosome showed a higher proportion of motile spermatozoa than those with damaged acrosome or with higher fluorescence intensity (Table 1). Within motile spermatozoa, those with the highest fluorescence intensity

showed signs of hyperactivation, as they were faster and had more vigorous movement than normal spermatozoa, whereas those with damaged acrosome were slower and exhibit weak movement (Table 1).

A significant positive correlation was observed between sperm membrane integrity determined with the AO/IP test and with the ISAS®3Fun test. A positive correlation was also found between sperm with intact membranes and those with intact acrosomes. Correlations between total motility percentage and membrane or acrosomal integrity, despite being significant, were weak (Table 2).

4. Discussion

Sperm quality studies conducted to date in order to estimate the potential fertility of semen have been based on the evaluation of different sperm quality tests assessed independently and combined only by using statistical approaches (Santolaria *et al.*, 2015; Sellem *et al.*, 2015). In animal breeding, the final goal is the setting of thresholds to determine which ejaculates and at which concentration are suitable for AI. However, the complexity of analysis often makes these methods impractical, and most AI centers are still using simplified approaches evaluating few parameters in a single test. The integrative method described in this paper allows us to establish a single threshold combining different parameters of semen quality. This threshold will determine the proportion of spermatozoa with the potential ability to reach and fertilize the oocyte, and could be also a good model for its application in human reproduction.

The new method enables a clear distinction of spermatozoa with intact plasmalemma and acrosome, which are essential for sperm function. An intact plasma membrane is required for steady metabolic functions, capacitation, oviduct cell interactions, ova binding processes and acrosome reaction (Yániz *et al.*, 2013a). The acrosome reaction is

a key step for a successful fertilization, as the acrosome contains enzymes enabling the passage of sperm through the zona pellucida (Yanagimachi, 1994; Yániz *et al.*, 2014). Normally, acrosomes of capacitated spermatozoa react upon exposure to the zona pellucida, cumulus cells, or other substances associated with oocytes (Parrish, 2014). Various stressors during semen processing can cause damage to the acrosome membrane or can provoke a premature acrosome reaction that would hinder or prevent fertilization.

The distinction of live spermatozoa with increased fluorescence intensity of the acrosome and flagellum and changes in the motility pattern was also possible using the new method. These changes are compatible with sperm capacitation. The increase in fluorescence intensity of the acrosome and flagellum may be a consequence of the biochemical modifications associated with sperm capacitation (Yanagimachi, 1994). The observed changes in motility are concordant with the hyperactivated movement characteristics of capacitated spermatozoa (Yanagimachi, 1994). The fact that spermatozoa with increased fluorescence intensity were immotile in higher proportion than normal sperm could be explained by the increased sperm activity in this subpopulation, leading to a premature exhaustion that decreases the percentage of motile sperm.

Sperm hyperactivation and the ability to undergo acrosome reaction are two defining signs of sperm capacitation. This physiological process is a prerequisite for fertilization, but it must take place in the female reproductive tract, not during sperm cooling or storage (Del Valle *et al.*, 2010). It has been suggested that semen cooling may induce premature sperm capacitation (Watson, 1995; Maxwell and Watson, 1996; Gillan *et al.*, 1997; Bailey *et al.*, 2003), and its magnitude may affect fertilizing ability of semen. Consequently, the study of this parameter may be of interest in cryopreserved bull

semen. The new method opens the possibility of evaluating the different changes associated to sperm capacitation simultaneously cell by cell. Comparison between raw and post-thawed samples will be done to clarify this point.

The fluorescent sperm subpopulations described in this paper may have a correspondence with the sperm subpopulations described in different species by applying statistical analysis to the kinematic and morphometric parameters provided by the CASA system (Martinez-Pastor *et al.*, 2005; Nunez Martinez *et al.*, 2006; Dorado *et al.*, 2010; Yániz *et al.*, 2015a; Yániz *et al.*, 2015b; Santolaria *et al.*, 2016; Valverde *et al.*, 2016; Yániz *et al.*, 2016). The use of these techniques allows classification of the overall sperm population into separate subpopulations, grouping spermatozoa with similar kinematic or morphometric characteristics. The observed differences in sperm motility characteristics between fluorescent sperm subpopulations described here suggest a logical association with kinematic sperm subpopulations. A relation between the sperm subpopulations described in this paper and the morphometric sperm subpopulations is also possible, given the fact that the sperm membrane and acrosome integrity may have an effect on sperm morphometry (Yániz *et al.*, 2015b), and these parameters are used here to describe fluorescent sperm subpopulations.

Results of the present study revealed significant positive correlations among the different of studied sperm quality parameters. As expected, results of sperm membrane integrity using the ISAS®3Fun and AO/IP test were positively correlated, as both methods are based on increased permeability of damaged sperm plasmalemma to fluorochromes (Yániz *et al.*, 2013b). The finding that sperm viability was positively correlated with the acrosomal integrity was also expected, as observation of the images revealed the presence of more spermatozoa with acrosomal loss in the dead than in the live sperm populations. Finally, sperm membrane integrity was correlated with sperm

motility, in agreement with previous studies (Jeyendran *et al.*, 1984; Fraser et al., 2001; Mantovani *et al.*, 2002; Kirk et al., 2005; Yaniz et al., 2008b; 2011). This association may be explained by the fact that all spermatozoa with damaged plasma membrane were static. The plasmalemma of spermatozoa is essential for metabolic functions and sperm motility requires the transport of compounds across plasma membrane. Hence, the loss of integrity of this layer is incompatible with sperm motility. However, there is a variable proportion of immotile spermatozoa with intact plasma membrane, so the study of both parameters is justified.

5. Conclusions

It was concluded that the ISAS®3Fun is an integrative method that represents an advance in sperm quality analysis with the potential for improving fertility predictions. Future studies will be performed to study the evolution of these fluorescent sperm subpopulations under different physiological/technological conditions and to determine the predictive capacity of the method on the potential fertility of semen.

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Figure legends

Figure 1: Multi-parametric assessment of sperm quality in bull showing the presence of different fluorescent sperm subpopulations: intact membrane (blue), damaged membrane (red), intact acrosome (a), damaged acrosome (b). Scale bar=10µm.

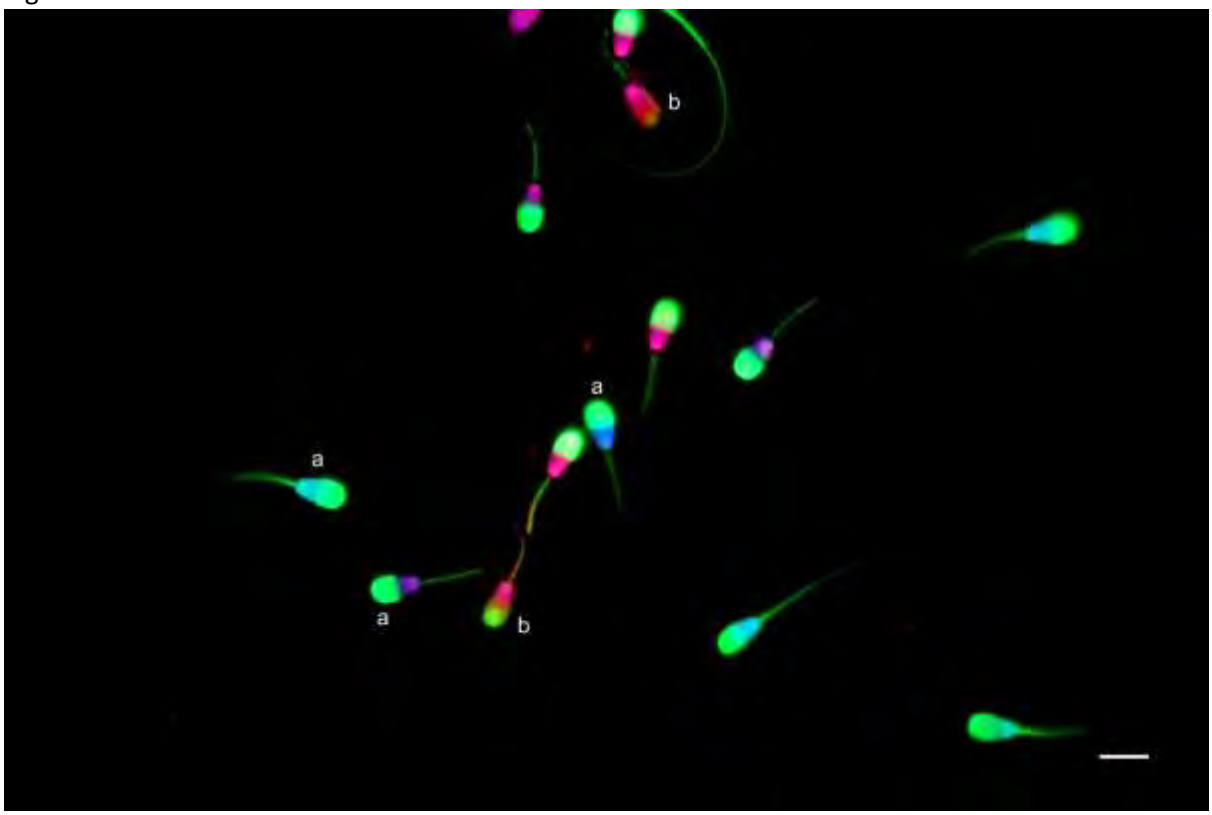
Figure 2: Bull sperm with intact plasmalemma and different fluorescent labeling patterns. (a) Intact acrosome and low fluorescence intensity; (b-g) increased fluorescence intensity and progressive acrosomal loss; (h) low fluorescence intensity and total acrosomal loss. Scale bar=5µm in (a) applicable to all micrographs.

Figure 3: Bull spermatozoa from a live sperm with intact structures to a dead sperm without acrosome. (a) Intact plasma membrane and acrosome; (b) plasmalemma with

increased permeability and normal acrosome; (c-e) damaged plasmalemma and progressive acrosomal loss; (h) total acrosomal loss. Scale bar=5µm in (a) applicable to all micrographs.

Figure 4: Long-time exposure photograph showing the trajectory of motile spermatozoa (a) and the presence of different fluorescent subpopulations of static spermatozoa (b). Scale bar=20µm.

Figr-1



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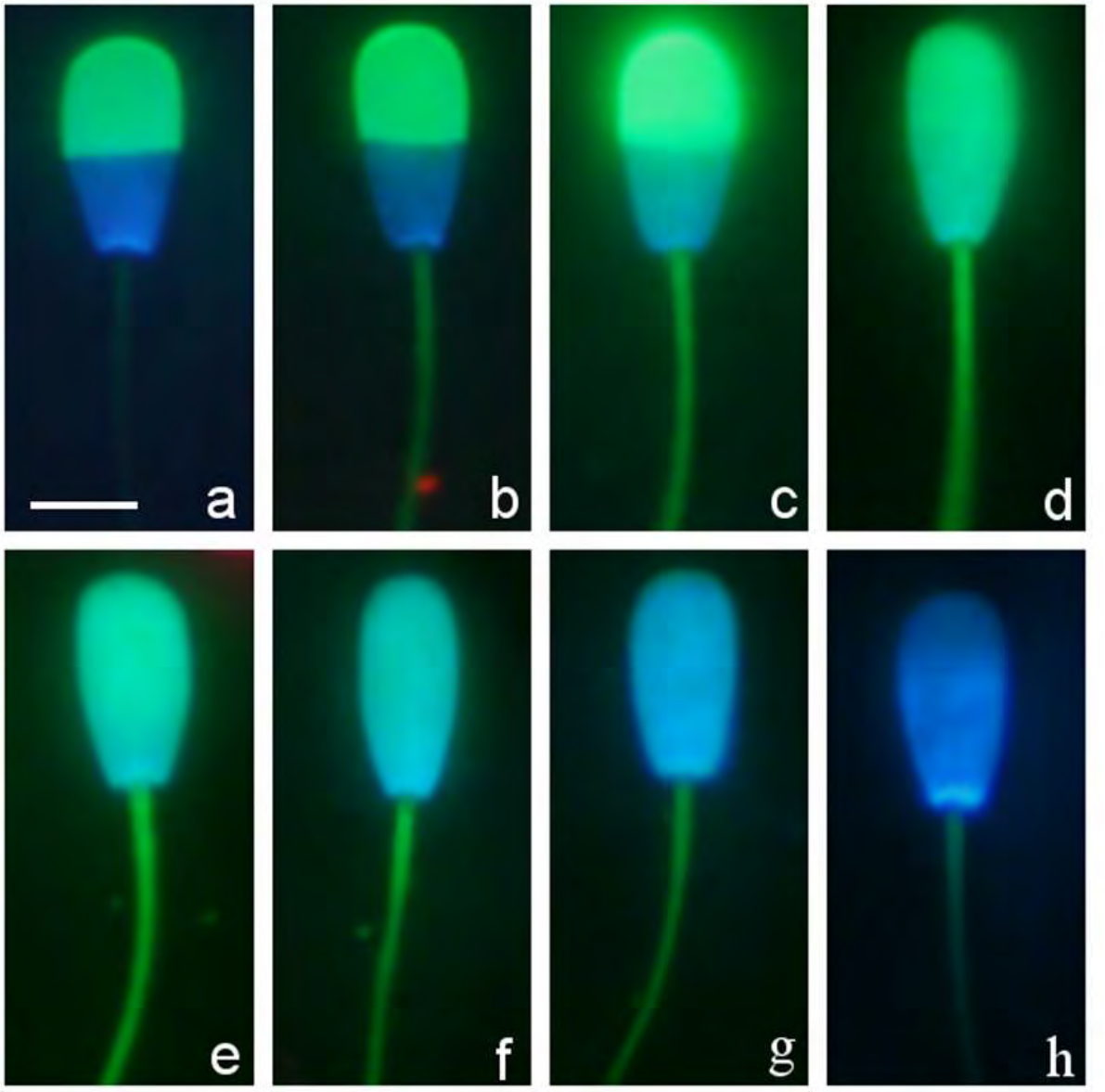

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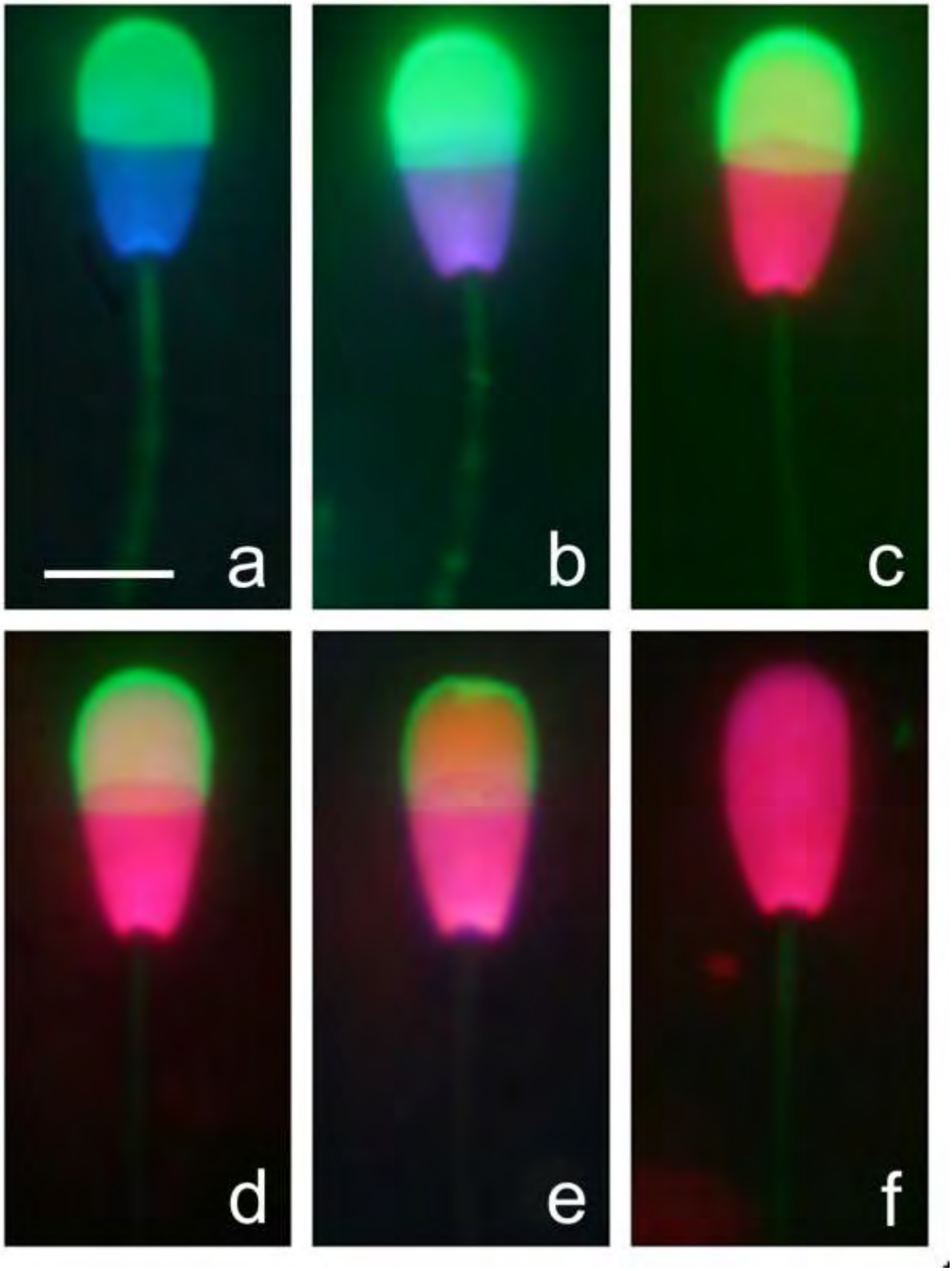

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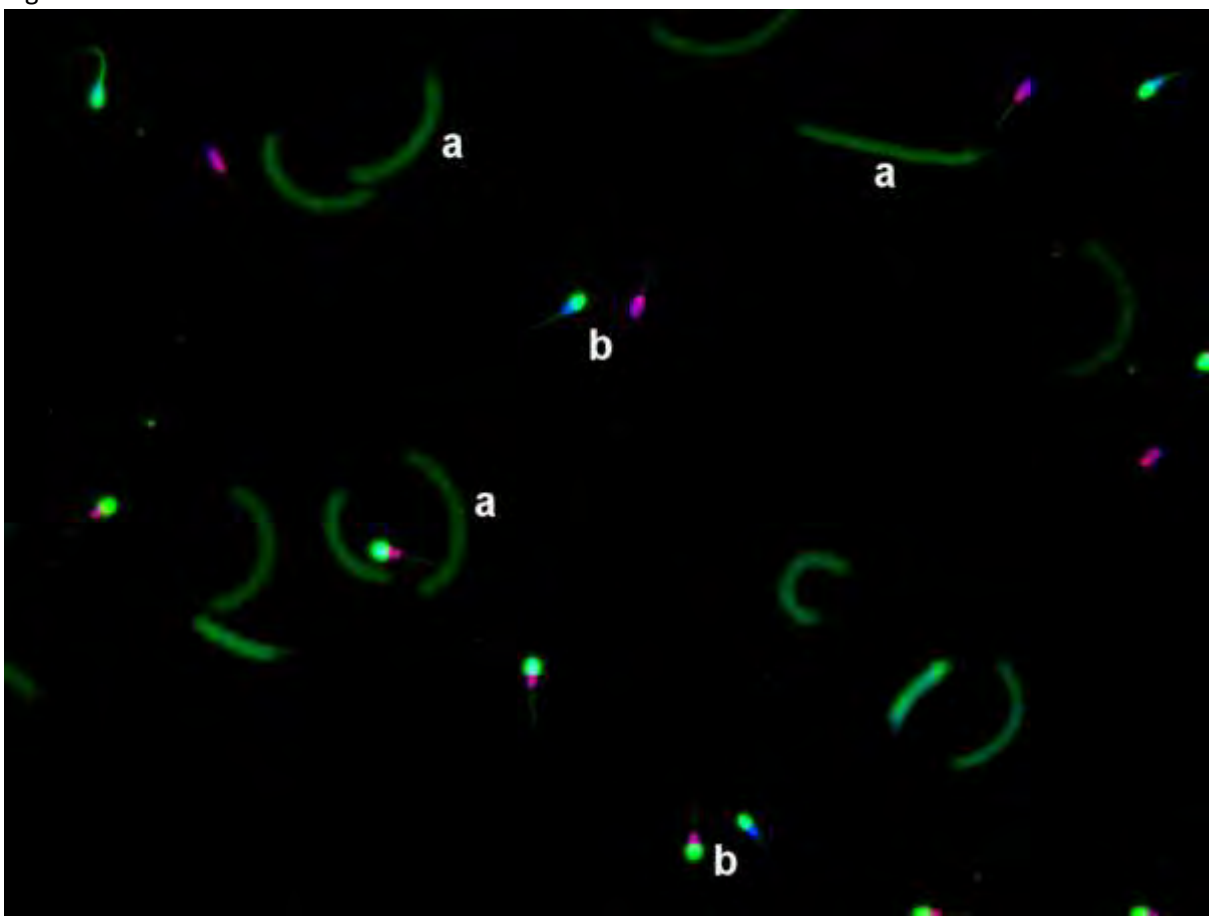

Table1. Differences in sperm motility parameters between the three fluorescent sperm subpopulations detected with the ISAS®3Fun that exhibit motility. Data, except MS, are represented as Mean ± S.D.

Sperm subpopulation	Motility parameters							
	MS (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	ALH (µm)
MNAN	83.23	105.00 ± 39.52[a]	59.71 ± 32.87[a]	81.79 ± 32.04[a]	57.17 ± 27.22[a]	71.13 ± 29.25[a]	74.19 ± 20.29[a]	5.35 ± 2.68[a]
IFI	59.09	183.65 ± 91.93[b]	141.06 ± 80.71[b]	164.45 ± 92.03[b]	76.38 ± 16.87[b]	86,59 ± 12.32[b]	87.91 ± 14.37[b]	9.25 ± 5.10[b]
MNAD	56.84	43.77 ± 18.73[c]	25.47 ± 17.02[c]	29.35 ± 20.42[c]	47.30 ± 27.70[d]	70.12 ± 23.73[a]	53.26 ± 29.05[c]	1.78 ± 1.17[c]

MNAN: sperm with intact membrane and acrosome; MNAD: sperm with intact membrane and damaged acrosome; IFI: spermatozoa with increased fluorescence intensity in head and flagellum; MS: sperm motility percentage; VCL: curvilinear velocity, VSL straight line velocity; VAP average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: lateral head displacement; BCF: beat cross frequency. [a-e] Superscripts denote differences between sperm subpopulations ([a-b, a-c, b-c]$P < 0.01$; [a-d]$P < 0.05$).

Table 2: Spearman's correlation coefficients between different sperm quality parameters in bull.

	Membrane integrity[1]	Acrosome integrity[1]	Motile sperm[2]
AO/PI	0.60**	0.62**	0.34**
Membrane integrity[1]		0.71**	0.47**
Acrosome integrity[1]			0.31*

AO/PI: membrane integrity determined with the acridine orange/propidium iodide stains; [1]Determined with the ISAS®3fun kit. [2]Determined with the ISAS® CASA-Mot system. Significant correlations at *P < 0.05 and **P < 0.01.