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1 **An update on boar semen assessments by flow cytometry and CASA**

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9 **Abstract**

10 In the quest for predicting fertility of an individual, enhancing semen handling,
11 dilution and storage protocols, and understanding the impact of environment and,
12 andrologists have changed their approaches to semen analysis. The technologies used today
13 are fast developing and readily implemented in research. Semen is one of a few naturally
14 occurring monocellular suspensions, so sperm function analysis by flow cytometry (FC) and
15 utilization of fluorochromes is an ideal technique for high throughput, objective and
16 accurate analysis. The complementary use of microscopical assessments by Computer-
17 Assisted Semen Analysis (CASA), where sperm cell parameters can be objectively assessed
18 is equally important. The objectivity and repeatability of these techniques have driven
19 research on the function, identification of heterogeneity and fertility of the ejaculate. The
20 wealth of knowledge obtained from the application of these powerful methods has changed
21 our view of the spermatozoon.

22 Although there is some application of these methods in the industry producing boar
23 semen for artificial insemination (AI) and to eliminate sires of sub-standard semen quality,
24 uptake of advanced methods is still slow. Instruments are becoming cheaper and technically
25 more user friendly. Standardization of methodology and optimization of instrument settings
26 is important for full implementation of these systems, including comparison between labs.
27 This review provides an update on two technologies: flow cytometry and CASA for
28 objective analysis of boar semen quality.

29

30

31 **Key words:** Boar; Spermatozoa, Motility, Flow cytometry, CASA

32 ## 1. Introduction

33 Fertility is a complex trait. Many physiological and cellular functions need to align
34 in space and time in both the female and male for success. There is no single fertility
35 measure for the seemingly simple cell, the spermatozoon, with an expected single objective
36 of "fertilizing an oocyte". To evaluate the fertility potential of a semen sample, we need to
37 determine its heterogeneity and its subpopulations and the "combined effective amount" [1].
38 Increasing the number of functional tests gives a more accurate account in our predictive
39 index of fertility potential. This approach is acknowledged and applied in the field of
40 andrology. Therefore, flow cytometry analysis (FC) and computer assisted sperm analysis
41 (CASA), are taken up in both industry semen evaluations and in research practice.

42 CASA, to objectively measure motility/kinematic patterns, and FC, to assess
43 functional compartments of the spermatozoon, have been used concurrently in semen
44 analysis for nearly half a century. With the integration of increasingly powerful computers,
45 these techniques have developed considerably, from humble beginnings of acetate sheets
46 and video freeze-frame to track individual sperm heads, and single wavelengths of
47 excitation and one to three emission detectors FC. These developments have offered
48 objective and reliable outcomes and increased our knowledge about the sperm cell, and their
49 subpopulations, with an extensive variety of applications. The combination of FC and
50 CASA enables multi-parametric assessment of sperm function.

51 The aim of this review is to present a summary of current stains and biomarkers
52 available for analyses of boar sperm quality and function using FC, and review new
53 protocols utilized for the assessment of spermatozoa. It is important to note that the methods
54 described in this review have not necessarily been optimized to the cell type and/or species.
55 This is followed by an update on CASA analysis in boar sperm analysis, and

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56 recommendations for its use. Prior to purchasing or using a flow cytometer or CASA
57 system, a solid understanding of the principles behind the technology are necessary for their
58 optimal use, which is outside the scope of this review.

59

60 ## 2. Semen assessment by flow cytometry

61 The uptake of FC by andrologists for the analysis of spermatozoa has been
62 phenomenal since its conception in 1968/69 by Wolfgang Göhde and Partec. The
63 advantages of FC over fluorescence microscopy include objectivity, sensitivity and high
64 numbers, providing higher accuracy. Spermatozoa from most animal species are ideal for
65 FC because they are naturally occurring monocellular fluid suspensions. Numerous reviews
66 and books have described the basic principles of FC.

67 A flow cytometer consists of one or more lasers emitting at specific wavelengths, a
68 flow cell that presents the cells in single file in a stream of fluid, and a range of filters
69 separating the emission signal. The two base signals are forward scatter (FSC) and side
70 scatter (SSC), commonly used to identify and gate cells for size and granularity,
71 respectively, and an overall impression and uniformity of the cell suspension. Therefore,
72 FSC/SSC on unstained/non-fluorescent samples is used to identify the sperm population and
73 gate out most of the cellular debris and bacteria. FSC/SSC cytograms of spermatozoa
74 typically shows an L-shaped distribution [2]. Samples can be stained for particular features
75 using fluorochromes. As the sperm flow through the flow cell, fluorochromes are excited by
76 a laser and the emitted light at different wavelengths is detected by a range of optical filters
77 and detectors. The original FC, introduced in the 1970s, consisted of a single laser, and a set
78 of up to three detectors. Today, the stability of solid state lasers enables the instrument
79 configurations to move from single to multiple lasers, and a comprehensive coverage of the

80 light spectrum, by up to 27 detectors. The largest FC instrumentation providers are
81 currently: BD Biosciences, Beckman Coulter, Millipore, Sigma, and Sysmex Partec. Most
82 research andrology laboratories would have FC capabilities as a basic routine tool.

83 A variety of different functional aspects of spermatozoa can be detected using FC.
84 These include sperm plasma membrane measures, acrosome integrity, mitochondrial
85 membrane potential, reactive oxygen species (ROS) and DNA damage. Advanced modern
86 instruments with multiple lasers, a broad range of detectors, and the ability to conduct multi-
87 parametric assays have opened new challenges in determining compensation for spectral
88 overlap and careful population gating. A variety of new fluorescent probes are available to
89 assess cells using both fluorescent microscopy and FC. Most stains have been developed for
90 analysis of somatic cells, and not spermatozoa. Therefore, validation before use in each cell
91 type and species is crucial, and new assays are tested for their impact or association with
92 fertility or sperm function [3]. Online tools to assess spectral overlap are becoming
93 increasingly important when planning an experiment and most vendors now offer these on-
94 line tools and apps freely, although often restricted to a vendor specific range of
95 fluorochromes; thus the use of a combination of these programs is advised. These tools also
96 help identify instrument requirements and configurations.

97 Semen contains predominantly spermatozoa, but cellular debris, white blood cells,
98 erythrocytes and bacteria can also be found, originating from the reproductive tract or as
99 additions from the environment at ejaculation. In addition to the classical approach of
100 selecting spermatozoa on cellular granulation and size using the FSC and SSC channels,
101 DNA dyes enable positive labeling to ensure spermatozoa are clearly distinguished from
102 cellular debris. A membrane-permeating fluorescent probe is often included as a positive
103 control or osmotic shock is used to permeabilize the spermatozoa, making them positive to
104 propidium iodide (PI) to measure non-sperm contamination [2].

105 The following sections summarize different cell function analyses using FC in boar 106 spermatozoa. In the light of fast developing methodology, with respect to both 107 instrumentation and availability of fluorochromes, computational rigor in biostatistics and 108 bioinformatics is increasingly important. New developments for sperm assessment have 109 been reviewed by [4, 5]; other reviews have covered the classical FC protocols used in 110 spermatology [6-8].

111 With advances in FC, it is increasingly important for researchers to include detailed 112 information about the analyses in their publications. Minimum Information about a Flow 113 Cytometry Experiment (MIFlowCyt) has been defined by the International Society for 114 Advancement of Cytometry. The guidelines are actively applied by journals, providing 115 guidance on conduct and interpretation of experiments using FC-based protocols [9].

116 *2.1 Sperm concentration*

117 Pioneering work for automated cell counting was conducted in the 1930s by Andrew 118 Moldavan on red blood cells. Due to their irregular shape, different approaches to FC have 119 been required to evaluate sperm concentration, including: 1) the quantification of a number 120 of events using a defined concentration of reference beads; and 2) true volume count [10]. 121 The BD FACSCount AF and the Coulter-counting technique were developed to determine 122 sperm concentration using beads and sperm viability stains (SYBR-14/PI) and were 123 validated on commercial boar AI stations [11]. These automated methods were shown to 124 provide an accurate and highly repeatable (CV 2.7%) determination of sperm concentration 125 in the boar AI dose [12], although manual methods, using haemocytometer, 126 spectrophotometer and in CASA systems (CASA-Conc) are also widely used.

127 Accurate discrimination of spermatozoa from other cell types and debris is essential. 128 Therefore, a range of cell permeable dyes can be used for this purpose. These are commonly

129 used in combination with other stains to identify the sperm cell and its specific attributes.
130 The most widely used method is by nuclear counterstaining [7]. In boar semen analysis
131 membrane-permeant DNA fluorochromes, SYBR-14 and Hoechst 33342 (H-42) are
132 standard. H-42, a highly permeable fluorescent stain, is best known for its use to
133 differentiate X from Y chromosome bearing spermatozoa based on binding to DNA.

134 *2.2 The sperm membrane*

135 Flow cytometry has been used to determine the proportion of viable (or membrane
136 intact) boar spermatozoa since the 1980s. Initial assays used fluorescein diacetate (FDA), 6-
137 carboxyfluorescein diacetate (CFDA) or 6-carboxymethylfluorescein diacetate (CMFDA)
138 and calcein acetomethyl ester (CAM) for identification of viable cells, by permeating an
139 intact live cell membrane. If the cells are viable, esterification occurs to a non-permeant
140 fluorescent compound retained in the cytoplasm. The combination of CFDA and the
141 membrane-impermeant nuclear stain PI was first used with FC in boar semen by Garner and
142 coworker [13].

143 Today membrane-permeant DNA fluorochromes are often used. For example,
144 SYBR-14 maximally absorbs at 488 nm and emits at 518 nm when bound to DNA and has
145 been used extensively in boar semen since its validation using FC by Garner et al. [14, 15].
146 A commercially available kit uses the combined stain SYBR-14/PI (Live-Dead Sperm
147 Viability Kit L-7011; Molecular Probes Inc., Eugene, OR, USA) to identify spermatozoa as:
148 unstained debris, live, moribund, and dead. The SYBR-14/PI assay uses two optical filters,
149 to assess a single function, and it is the most widely validated viability assay for boar sperm
150 analysis.

151 Newly developed DNA stains, such as the SYTO® dyes, can be used as alternatives
152 to SYBR14 and/or PI, depending on the fluorochrome characteristic: including cell

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153 permeability, fluorescence enhancement upon binding nucleic acids, DNA/RNA selectivity,
154 and binding affinity. Examples of SYTO® dyes used in FC protocols in boar sperm analysis
155 are identified in Table 1 [16].

156 For detection of further membrane characteristics, such as changes associated with
157 apoptosis, CFDA, ethidium homodimer-1 (EthD-1), the cyanine Yo-Pro (Yo-Pro-1) and
158 Hoechst 33258 (H-58) can be used. For example, Yo-Pro-1, a membrane impermeable
159 probe, enters the cell only after membrane destabilization indicative of damage or apoposis.
160 It has been used in a large number of studies on boar spermatozoa (examples in Table 1),
161 with a simple counterstaining [17] or in a more complex triple staining combination with
162 carboxy-seminaphtharhodafluor (SNARF)-1 and EthD-1 [18], where results are comparable
163 in correlation to Annexin-V/PI [18, 19] and other "apoptosis indicators" include EthD-1 and
164 H-42 [4].

165 The fluidity of the sperm membrane is often assessed using Merocyanine 540 (M-
166 540), a lipophilic molecule impermeant to intact cells which binds to the outer leaflet of the
167 plasma membrane. M-540 is often combined with Yo-Pro-1 to identify viable boar
168 spermatozoa with low membrane lipid disorder [20-22]. When the cell membrane is
169 damaged, phosphatidylserine flipping occurs as the phospholipids translocate from the inner
170 to the outer leaflet of the plasma membrane. This early sign of apoptosis can be monitored
171 by the calcium-dependent binding of Annexin-V conjugated with fluorescein isothiocinate
172 (FITC), used to determine sub-lethal cryo-damage in boar spermatozoa [8, 19, 23, 24].

173 A number of new fixable dyes, appropriate to test membrane characteristics have
174 entered the scene of FC analysis. They are available in a multitude of different colors, with
175 narrow spectra, making them suitable for multicolor experiments. These include the amine
176 reactive dyes (ARDs), which interact only with the plasma membrane surface amines of

177 intact cells. If the membranes are compromised, ARDs also react with intracellular free
178 amines, increasing the amount of labeling, including the Zombie®, Ghost Dyes® and other
179 fixable viability stains. Combinations of fixable ARDs, such as Live/dead Zombie Green®
180 and MitoTracker Deep Red®, have been used for stallion spermatozoa. The advantage of
181 fixable stains is the ability to delay determination of sperm viability and mitochondrial
182 membrane potential [25].

183 *2.3 Acrosome status*

184 Plant lectins labeled with fluorescein-labelled (FITC), specifically *Pisum sativum*
185 (edible pea) agglutinin (PSA) and *Arachis hypogaea* (peanut) agglutinin (PNA), have been
186 extensively used in assessing acrosome integrity in spermatozoa. PNA has been preferred
187 over PSA, because PNA-FITC labelling is restricted to the acrosome, binding to β-galactose
188 moieties, which are associated with the outer acrosomal membrane. PSA-FITC is less
189 specific, binding to the α-mannose and α-galactose moieties and staining the acrosomal
190 matrix, but also the flagella and head. Labelling with plant lectins only occurs when the
191 plasma membrane and outer acrosomal membrane are damaged, allowing the lectins to
192 interact with the acrosomal carbohydrate moieties, and do not provide any information on
193 the molecules that actually function during the fertilization process. A large number of
194 studies in boars have used the PNA-FITC/PI and PSA-FITC/PI combination [22, 24],
195 identifying sperm subpopulations with intact plasma membranes and intact acrosomal
196 membranes [26]. PNA has also been labeled with Alexa Flour 488 (PNA-AF488; [27, 28]),
197 cyan-green color, or Alexa Fluor 647 (PNA-AF647; [16]) in the far-red emission spectrum,
198 circumventing some of the issues which occur with emission spectra and optical filters
199 fluorescein labeling. Multi-function staining methods which include acrosomal status
200 indicators are gaining popularity (see Table 1: [8, 29-31]).

201 A number of proteins that have been identified in the human and mouse sperm
202 acrosome, which could also be used as molecular biomarkers to assess sperm acrosome
203 status [32]. FITC-conjugated goat anti-mouse immunoglobulin bound to CD46 antibody has
204 been used to determine acrosome status in humans [33]. Other protein markers related to the
205 acrosome reaction are likely to be identified and used for the development of specific
206 antibody FC assays. There have been discussions on the optimization and standardization of
207 acrosome reaction induction protocols in boar spermatozoa as part of the assessment criteria
208 for semen quality [34], in order to standardise accepted parameters so they can be included
209 in industry QC procedures.

210 *2.4 Mitochondria and oxidative stress*

211 With increased interest in sperm mitochondrial biology, and seminal plasma and
212 sperm interactions, the presence and actions of ROS in semen needs to be determined. FC is
213 among the technologies that have been applied to assess sperm mitochondrial biology and
214 mitochondrial oxygen consumption [35]. Different fluorescent probes have been used to
215 detect changes in sperm mitochondrial membrane potential (MMP) using FC, discriminating
216 cells with active and non-active mitochondria. It is not clear whether changes in MMP are
217 due to a primary marker of sperm capacitation, but motile boar spermatozoa have higher
218 mitochondrial activity than immotile ones [36]. The commonly used fluorescent dyes to
219 detect sperm mitochondrial activity in boar spermatozoa include Rhodamine 123 (R123,
220 [37]), 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolyl-carbocyanine iodide (JC-1,
221 [22]), and increasingly MitoTracker Deep Red 633 (MTdr [38]). Lesser known stains
222 include Tetramethylrhodamine methyl ester perchlorate (TMRM) and 3,3′-
223 dihexyloxacarbocyanine iodide (DiOC6(3)). TMRM is traditionally a somatic cell stain
224 which accumulates in the mitochondria, similar to JC-1 [39]. DiOC6(3) is a cell-permeant,
225 green-fluorescent, lipophilic dye that is selective for the mitochondria in live cells [40].

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226 Other MMP sensitive probes include MitoTracker® Orange CMH2TMRos, a derivative of
227 the orange-fluorescent tetramethylrosamine, and MitoTracker® X-Rosamine CM-H2XRos,
228 a red-fluorescent X-rosamine. These only fluoresce after oxidation, indicating oxidative
229 respiration, which is only relevant if the mitochondria are functional [41]. The probes may
230 be suitable to discriminate spermatozoa with deteriorated mitochondria.

231 Fluorescent probes for measuring free radicals in mitochondria can predict the
232 quality and function of the organelle. The two probes, 2′, 7′-dichlorodihydrofluorescein
233 diacetate (H_2DCFDA) and hydroethidine or dihydroethidium (DHE) have been used to
234 detect H_2O_2 and superoxide, respectively, in boar spermatozoa [42]. H_2DCFDA is stable,
235 non-fluorescent cell-permeable probe, which de-esterifies intracellular leaving impermeant,
236 non-fluorescent, H_2DCF. In the presence of intracellular H_2O_2 it forms 2′,7′-
237 dichlorofluorescein (DCF) which emits a fluorescence at 530 nm [42-45]. Other variants of
238 this probe include CM-H_2DCFDA, which is better retained in live cells than H_2DCFDA, and
239 Carboxyl-H2DCFDA (carboxylated H_2DCFDA analog, [46, 47]). The freely permeable
240 non-fluorescent stain DHE is oxidized by superoxide, and to a lesser extent other ROS, to
241 ethidium, which binds to DNA and becomes fluorescent [45, 48]. The stain MitoSOX Red
242 (MSR) is mitochondrion-targeted and a cationic derivative of DHE [36].

243 Simultaneous FC assessments of caspases 3 and 7 activity, viability, and ROS have
244 been conducted in stallion spermatozoa [49]. In boars, a caspase FITC-VAD-FMK in situ
245 marker has been used to assess active caspases in boar semen by FC [50] where the FITC
246 conjugated cell permeable caspase inhibitor peptide was used to identify activated caspases,
247 indicating apoptosis. Within the last decade, monitoring peroxidative damage in
248 spermatozoa has become possible using BODIPY(581/591)C(11) in bull and boar semen
249 [51]. The probe incorporates into cells and undergoes a spectral emission shift, which is
250 uniquely very small, when attacked by reactive oxygen metabolites, indicating exposure of

251 phospholipids to ROS and quantifying lipid peroxidation. The color of the probe changes
252 according to its oxidation state (none-peroxidized membrane: red; and lipid peroxidized
253 membrane: green) [42, 48]. Bis-BODIPY-FL C(11) has been used in boar spermatozoa
254 measuring phospholipase activity by the presence of endogenous PLA1 or PLA2 [42]. In
255 general, the levels of lipid peroxidation are relatively low [45, 48, 52], but degradation
256 products from lipid peroxidation, like 4-hydroxynonenal (4- HNE), acrolein (ACR) and
257 malondialdehyde (MDA) can also be targeted in FC protocols [4].

258 *2.5 Calcium uptake and intracellular pH*

259 Flow cytometry can be used to measure ion indicators, such as calcium [Ca^{2+}] uptake
260 before and after in vitro capacitation, measuring the responsiveness of spermatozoa to the
261 capacitation stimulus bicarbonate. The Fluo-3 AM (Fluo-3), was first used to assess Ca^{2+}
262 uptake in the midpiece region of boar spermatozoa by FC over 25 years ago [53] and has
263 been used to determine the response in a capacitating medium containing bicarbonate,
264 compared with a non-capacitating medium [54-57], as well as to study the function of the
265 CatSper channel in relation to capacitation in boar spermatozoa [58]. Rhod-5N-AM (Rhod5)
266 has a lower calcium affinity than Fluo-3 and cellular localisation of stained Ca^{2+} in the
267 acrosomal and post-acrosomal regions [56]. Fluo-4 AM has been used to assess cytosolic
268 calcium in boar spermatozoa [8] to detect changes of calcium influx. In a multilaser
269 instrument configuration, Indo-1 AM, excited by a 405 nm (UV) laser [59], Fura-2, and
270 eFluor™ 514 Calcium Sensor Dye [7] can be used to detect calcium increases during
271 capacitation in boar spermatozoa, although these are yet to be extensively used for boar
272 sperm assessments.

273 The intracellular pH of the spermatozoa has also been used to indicate viability and
274 function. Intracellular pH has been associated with sperm motility and the acrosome

275 reaction [60]. 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester
276 (BCECF AM; [53]) diffuses through the plasma membrane, hydrolyzed by esterases
277 released into the cytoplasm and, when excited, the fluorescence intensity is dependent on
278 pH. CDCF diacetate can determine compartmental integrity [53]. Carboxy-
279 seminaphtharhodafluor-1 (SNARF-1; [22]), also hydrolyzed by esterases present in viable
280 cells, leaving SNARF-1 trapped within the cell. Lysosomal probes, such as LysoSensor™,
281 have been designed for labelling acidic organelles in somatic cells, but there have been few
282 trials in domestic animals [61].

283 *2.6 Detection of cell surface changes*

284 The sperm surface contains and displays various proteins with specific functions,
285 often obtained after post-translational modification, which are involved in key interactions
286 in the female tract and in fertilization [62]. These proteins can be used as markers for male
287 infertility, and detected changes used for optimization of semen handling protocols [63]. FC
288 can identify specific biomarkers on the sperm surface to evaluate the subpopulation of
289 spermatozoa with the desired attribute. Most research to date has been limited to human
290 spermatozoa and those of domestic species other than pigs [5]. However, with increased
291 knowledge of the membrane complexity and proteome analysis of the sperm surface [64],
292 for example in good versus bad responders to cryopreservation [65], specific targets are
293 being identified to improve boar semen quality [66]. Some specific examples include
294 SPTRX3/TXNDC8, ubiquitin, and postacrosomal Sheath WWI Domain Binding Protein
295 (PAWP), which have been validated as negative biomarkers of human and/or animal male
296 fertility [67], with potential to be useful in boar semen analysis.

297 Detection of protein tyrosine phosphorylation, which in the boar has been associated
298 with capacitation [68], has been used to identify subpopulations by FC, using an anti-

299 phosphotyrosine antibody conjugated to FITC [69]. In boar semen, the sulfhydryl group
300 content of proteins from the sperm surface can be detected using 5-
301 iodoacetamidofluoresceine (5-IAF) [70]. The biomarker ubiquitin can also be detected using
302 MK-12-3 anti-ubiquitin monoclonal antibody [71]. Arachidonate lipoxygenase ALOX15
303 has been detected using FC in boar semen, using Anti-15-LOX antibody and goat anti-rabbit
304 IgG-FITC [72].

305 *2.7 Chromatin defects*

306 The integrity of sperm DNA and chromatin has long been thought to be important
307 for early embryonic development. A number of stains have been used to assess chromatin
308 defects, such as the sperm chromatin structure assay (SCSA – using acridine orange (AO)),
309 terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labelling
310 (TUNEL – using FITC conjugated with 2′-deoxyuridine 5′-triphosphate (dUTP)) and the
311 sperm protamine deficiency assay (SPDA – using chromomycin A_3 (CMA3)). Details of the
312 available assays are provided in Table 1.

313 The SCSA, originally developed for human semen and used in the human fertility
314 clinics, has been used in boar semen to analyze DNA fragmentation index (DFI) [73]. The
315 assay is based on the metachromatic properties of the fluorochrome AO [74]. DFI and the
316 percentage of spermatozoa with an abnormally high DNA stainability, have been shown to
317 correlate with fertility in a heterospermic competitive study [73] and litter size [75, 76].

318 The TUNEL assay is based on the identification of DNA breaks by addition of
319 template-independent DNA polymerase called terminal deoxynucleotidyl transferase (TdT)
320 to the 3′hydroxyl (OH) breaks-ends of single and double stranded DNA. FITC is conjugated
321 with 2′-deoxyuridine 5′-triphosphates (dUTPs) and the fluorescent signal measured by FC is
322 directly proportional to the sperm DNA fragmentation [77]. The spermatozoa with

323 fragmented DNA present an intense green nuclear fluorescence, in comparison with
324 spermatozoa with intact DNA showing low green fluorescence.

325 Mature mammalian spermatozoa have a compacted and stable nuclear structure
326 involving protamines and histones. In the boar and bull, protamine 2 is transcribed and
327 translated on very low levels [78], hence the packaging of the chromatin in the boar is
328 mainly protamine 1 [79]. Protamine 1 has a high level of cysteine residues, which form
329 disulfide bridges assisting in sperm nuclear condensation in the maturing gamete. Methods
330 have been adapted for FC determining chromatin thiol status and compaction using
331 monobromobimane [80] and chromomycin A3 (CMA3), respectively. CMA3 is useful for
332 the detection of protamine deficiency in human spermatozoa [81, 82], and has been used to
333 identify correlations between sperm protamine deficiency and sperm DNA fragmentation by
334 FC [83, 84]. Our group recently developed a sperm protamine deficiency assay based on FC
335 utilizing both UV and visible spectra of CMA3 to assess the deficiency of protamines on
336 bull sperm DNA [85].

337 *2.8 Multiparametric flow cytometry*

338 Multi-laser and >3 detector FC configurations, using combinations of more than two
339 or three stains, have become common. Concurrent innovations in fluorescent probes with
340 narrower emission-spectra and improved analytical software, using multiple staining
341 procedures for simultaneous assessment of functional attributes in the same cell population,
342 enable the identification of distinct cell subpopulations to further characterize samples.
343 Multiparametric flow cytometry avoids the splitting of semen samples into multiple aliquots
344 to analyze for a single sperm trait per aliquot. This may assist also to identify the population
345 of ideal spermatozoa with all required attributes for successful fertilization and normal
346 development of the embryo. A small number of double and triple stain procedures have

347 been mentioned throughout this review, such as multiparametric methods with four-fold
348 stains to evaluate plasma, acrosome and mitochondrial membrane potential, while excluding
349 cell debris [69]. With the complex configurations of instrumentation and new stains,
350 counterstaining using SYTOX™ dyes, DRAQ® dyes, and 7-aminoactinomycin D (7-AAD),
351 will enable better discrimination of functions and optimized methods. The practicality of
352 using traditionally difficult fluorochromes such as M540 and JC-1 [36] increases with multi-
353 laser instruments. The narrowing of bandpass optical filters further enables complex
354 multiparametric protocols using the many available fluorochromes such as resazurin [86],
355 MitoSOX Red [87], To-Pro3 [88] and SYTO® dyes [13, 47, 89, 90].

356 Complex multiparametric studies using a large number of colors, will require an increase in
357 the number of 2D plots for every marker combination. To cope with the complicated data
358 analysis required, automated computational methods have been developed to identify
359 populations in multidimensional flow cytometry [91]. Babamoradi et al. applied automated
360 multi-dimensional data alignment/preprocessing and identification of FC boar sperm
361 populations using a density-based clustering method [92]. However, the recent development
362 of ACCENSE (Automatic Classification of Cellular Expression by Nonlinear Stochastic
363 Embedding) software, provides a more holistic automated approach
364 (http://www.cellaccense.com/), which has been applied to stallion spermatozoa where more
365 than four fluorochromes were used simultaneously [49], described in a recent review [5].
366 The implementation of multiparametric and computational flow cytometry has great
367 potential to create new discoveries and increased knowledge in the field of semen
368 assessment.

369 *2.9 Future of flow cytometry in sperm function analysis*

370 Traditional FC is being bypassed in research by imaging flow cytometry (IFC),
371 which captures multichannel images of hundreds of thousands of single cells in a short time.
372 The technology couples traditional FC with microscopy, allowing for morphometric
373 characterization of cells in addition to fluorescent imaging. Over the last 10 years IFC has
374 been used in studies on both human spermatozoa [93], and recently on those from other
375 mammalian species [94]. IFC and epifluorescence microscopy were used to trace the sperm
376 zinc signature in different mammalian species, including boar spermatozoa using Zn-probe
377 FluoZin™-3 AM (FZ3) [95]. The same research group has also demonstrated assessment of
378 the ubiquitin-proteasome system during boar sperm capacitation, determining changes in
379 proteasome compartmentalization, subunit composition and post-translational modifications
380 of the 26S proteasomal subunits. They used primary antibodies for 26S proteasome subunits
381 [96].

382

383 **3. Semen assessment by CASA**

384 CASA encompasses assessment of sperm motility/kinematics, and more recently
385 sperm morphological features and characteristics. The first CASA systems were developed
386 in the late 1970s to early 1980s to minimize the subjectivity of the manual assessment of
387 motility (reviewed by [97, 98]). Today CASA provides an objective, independent
388 interpretation, with detailed measurements on sperm motility, based on optical microscopy
389 and 2D videomicrography. The acronym CASA: computer-assisted sperm analysis, has
390 outgrown itself. The name is uninformative, since it could refer to any aspect or
391 characteristic of a population or of an individual spermatozoon. More appropriate names
392 indicating what is actually being assessed are being adopted, including CASA-Conc
393 (concentration), CASA-Mot (motility and/or kinematics), and CASA-Morph (morphology

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394 and/or morphometry) [99]. Other abbreviations for the later term include CASMA or
395 ASMA [100].

396 The most validated and traditional parameter measured by CASA is motility, which
397 is a fundamental functionality of a CASA system. Modern CASA systems have developed
398 to encompass improved algorithms to assess additional sperm characteristics such as
399 concentration, morphology and viability. The portability of these systems is also a
400 considerable advantage for application and implementation to industry. Most major
401 andrology laboratories have a CASA system, including larger boar semen processing
402 facilities [98]. A number of systems are available, but the main ones involved in veterinary
403 applications are: Minitube (Androvision), Hamilton Thorne (IVOS, CEROS), Microptic
404 (SCA), PROiSER R+D (ISAS), Biophos (Qualisperm), Medical Electronic Vision (SQA-
405 vision), (CRISMAS) and DITECT (SMAS).

406 CASA systems with specific boar sperm CASA-Mot programs are limited to
407 Androvision, IVOS, CEROS, SCA and ISAS. These systems have mostly evolved from
408 "black boxes" to having additional (and often user-adjustable) operational parameter
409 settings, including direct access to the microscope which allows better application to
410 research, portability and adjustments for different species [101]. The relatively high cost of
411 these products has resulted in the development of open-access and adaptable plugins, like
412 Image J CASA. The National Institutes of Health originally developed a CASA plugin for
413 the Image J software for fish spermatozoa [102], which has been modified for other
414 domestic animals (bull [103] and stallion [104]). Another open-source sperm tracker
415 software (Sperm Motility Tracker, V1.0) has been validated for ram and buck sperm
416 motility [105]. With user background knowledge and understanding, these new software
417 packages are advantageous due to flexibility and low cost [106, 107].

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418 The CASA terminology for motility is well established and has been described in
419 detail in reviews [98, 106]. Briefly, the lines and paths are constructed using algorithms
420 connecting the centroids for individual sperm heads, which provide its actual trajectory; the
421 curvilinear path. The time-averaged velocity along this trajectory is the curvilinear velocity
422 (VCL; µm/s). The average path and time-averaged velocity along this trajectory is average
423 path velocity (VAP; µm/s). The straight-line path from the first to last position of a sperm
424 head, and its velocity along this trajectory is the straight line velocity (VSL; µm/s). The
425 linearity (LIN; VSL/VCL ratio; %), straightness (VSL/VAP ratio; %) and oscillation (WOB;
426 VAP/VCL ratio; %) is often calculated based on these velocity parameters. At each point,
427 the deviation of the centroid from the average path is termed the amplitude of lateral head
428 displacement (ALH; µm). Where the curvilinear path intersects the average path, and the
429 number of intersections is termed beat-cross frequency (BCF; Hz). Some instruments also
430 routinely record a range of other kinematic outputs, including distance of average path
431 (DAP), straight line distance (DSL), and curvilinear distance (DCL). The default standard
432 description of settings for boar sperm are often set to VAP >20µm/s, and VSL ≥40 µm/s,
433 with a threshold for total motility may be set at a value around 70%. In research, the
434 multiparametric nature of CASA data increasingly requires complex/sophisticated statistical
435 analysis, routinely conducted with methods of cluster analysis. Data-defined clustering of
436 sperm subpopulations is extensively used in an attempt to make biological sense of the
437 standard descriptors [6, 108]. The use of unsupervised clustering (with non-hierarchical,
438 hierarchical and two-step methods) and advanced supervised methods, based on machine
439 learning, have recently been reviewed [109].

440 Sperm morphometry has now become common in the suite of parameters measured
441 by CASA platforms. The main parameters include measurements of the sperm head (length,
442 width, perimeter and area), cytoplasmic droplets identification, midpiece and tail (length and

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443 abnormalities). Most morphometry systems were initially developed for human spermatozoa
444 based on Strict Kruger-Tygerberg sperm normality criteria and WHO standards [110]. Boar
445 sperm head shape is classified as homomorphous compared to other species with more
446 heteromorphous sperm heads [100]. The importance of subtle variations in boar sperm
447 morphology has recently been reassessed [111] with growing evidence that morphometric
448 differences among sperm may affect fertility or dictate cryopreservation outcomes.
449 Naturally, boar spermatozoa with abnormal morphology are discriminated against after
450 deposition in the female [112]. Subtle defects in the midpiece and tail are also associated
451 with production outcomes such as the presence of cytoplasmic droplets negatively correlates
452 with both pregnancy rate and litter size [113]. Sperm head morphometric data vary between
453 boars and breeds. These variations do not appear to be correlated with sperm DNA integrity
454 [114] but perhaps the presence of cytoplasmic droplets is more indicative of altered
455 morphometric characteristics of the boar sperm head and chromatin instability [115].

456 CASMA usess three types of microscopy, conventional bright field illumination
457 (requiring staining), phase contrast (using wet-mounts) and fluorescence (requiring a
458 fluorescent probe). The products developed specifically for the boar semen industry measure
459 sperm concentration, motility and dose calculations, with some measures of morphology
460 (summarized in Table 2). Image J open-access software can also be applied to measure boar
461 spermatozoa only in a semiautomatic way developed from fluorescence staining, followed
462 by imaging and image analysis [116] or non-fluorescent staining such as SpermBlue [117].

463 The ability of these new CASMA systems to update and deploy morphological
464 classifications of sperm subpopulations, would enable further advancement of
465 morphological analysis in research as well as integration of their use in industry semen
466 assessment procedures.

467

468 ## 4. Future of CASA in sperm analysis

469 With improved computer systems, algorithms and image capture, CASA systems
470 have greatly improved in accuracy. The major limitation of the technology is its reliance on
471 2D optical microscopy. For boar sperm motility assessment, a ×10 or ×20 objectives is
472 commonly used, with a resulting theoretical depth of the field of 8.5 or 5.8μm, respectively
473 [98]. When considering the length, helical motion of the sperm tail, and the effect of
474 restricted movement close to the glass surface, the chamber depth should be 10 times greater
475 than this, which is not possible to achieve using traditional optical microscopy. Hence, there
476 are limitations to the type of the measurement that can be obtained for natural motility
477 patterns in the traditional CASA optical microscopy systems.

478 Three dimensional tracking has detected new human sperm motility patterns and
479 trajectories using lens-free holographic microscopy (HM) [118]. This novel approach uses
480 Multi-Illumination Single-Holographic-Exposure Lensless Fresnel (MISHELF) microscopy
481 [119]. MISHELF is a coherent imaging technique without normal lenses, but rather a digital
482 sensor that records a magnified hologram of the cells in motion. Three-dimensional motility
483 tracking, by digital HM in combination with a fast camera frame rate of 100 fps, and a deep
484 100 μm chamber, allows for analysis of the natural three-dimensional pattern of sperm
485 movement, and has been applied to boar spermatozoa [120]. The system is in prototype, but
486 it is likely to change the way andrologists view and measure sperm motility and trajectories,
487 including patterns of hyperactivation, in the future.

488 Another approach is the use of mobile phone microscopes for in-field diagnostics.
489 These have been made possible by improvements in the imaging capabilities of electronic
490 devices, including smartphone-based CASA applications for sperm analysis. One

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491 smartphone system, iSperm, consist of a lens, heat stage and disposable microfluidic chip,
492 which is directly attached to a phone or tablet, and functions on Apple iOS8 (Aidmics
493 biotechnology, Taipei, Taiwan). iSperm allows the evaluation of sperm concentration and
494 the percentage of motile spermatozoa, but not motile sperm trajectory [121]. These
495 simplified systems may be of less relevance in research, but due to their portability,
496 affordability and convenience, may be practicable on-farm.

497 There are two new CASA-like instruments based on fluorescence microscopy with
498 imaging: the NucleoCounter® SP-100™ (Chemometec) device for semen analysis, and the
499 Seminal Quality System (SQS) Horizon 2020 (Zoitechlab). These systems require a separate
500 instrument, staining in either cassettes or special slides, and measure concentration and
501 sperm viability and, in the case of the SQS, some aspects of sperm morphology. The
502 instruments do not assess motility and are therefore not traditional CASA systems, nor do
503 they incorporate FC. Both systems measure sperm concentration and plasma membrane
504 integrity using florescent probes. The semen analysis is conducted automatically, offering a
505 degree of both objectivity and speed, allowing for better semen quality analysis in an
506 industry setting.

507

508 ## 5. Conclusions

509 Technologies for measurement of sperm attributes are changing dramatically with
510 the improvement of optics and instrument capabilities. Developments in both FC and CASA
511 instrumentation are fast and ongoing. Our view of the spermatozoon is evolving and so is
512 the hunt for identification of the perfect spermatozoon or sperm population. The use of
513 semen assessment to not only determine when a sample is likely to be less ideal or
514 subfertile, but also to predict fertility or fertilizing potential, is opening up new insights into

515 functional aspects of these cells. In the boar semen preservation industry, the requirements
516 for a normal ejaculate are often stipulated as a minimum of 75% morphologically normal
517 spermatozoa, total motility >70%, and a total number of $\geq 20 \times 10^9$ spermatozoa, with a total
518 number of spermatozoa per dose of approximately 2.0 billion [122]. More vigorous quality
519 measures are rarely used in the industry.

520 To date, most FC applications in boar sperm analysis measure one functional
521 fluorochrome with a DNA counterstain such as PI to omit dead spermatozoa. A number of
522 these assays are applied to aliquots of the same sample. These assays are simple, distinct
523 emission maxima with minimal spectral overlap [7], but this is laborious. With the increased
524 availability and accessibility of multilaser and filter flow cytometers, an increased number
525 of narrow spectra fluorochrome probes and the development of automatic computational
526 methods, validation of multi-parametric protocols and their applicability to the industry are
527 likely to increase.

528 Current knowledge and new evaluation assessments can help identify subfertile
529 boars, before the young boar is incorporated into an commercial AI program [123],
530 removing individuals that may negatively impact general herd fertility, and hence increase
531 production efficiency. Current developments and reductions in the cost of CASA and FC
532 instrumentation mean that cost-effective systems are available for AI centers [123].
533 Multiplex FC and CASA protocols have been shown to assist in the breeding soundness
534 examination of sires, identifying sub- and in-fertile animals, as well as enabling the
535 optimization of semen collections and preparation methods, environmental effects and in
536 toxicological studies beyond andrological research and into the pig AI industry.
537 Furthermore, in the "multi-omics" era, there is an increased demand for reliable
538 confirmative methodologies, to enable molecular data to be linked with peripheral

539 physiological traits, genetic variants and phenotypic identification, particularly in the search
540 for “biomarkers” [124].

541

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904 **Table Legends:**

905 Table 1: Commonly used fluorochromes in sperm function assays by flow cytometry (FC).

906 Table 2: Commercially available CASA systems with boar semen specific configurations.

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Target/Assay	Stain	Emission color	Common use	References
Nucleic acids	Hoechst dyes	Ultraviolet	Cell counting and/or cell identification; DNA concentration	Example [4]
	Propidium iodide (PI)	Red	Most commonly used counterstain for moribund and dead spermatozoa	Example [7]
	Ethidium homodimer	Deep red	Membrane impermeable dye, alternative to PI to avoid spectral overlap.	[4, 18]
	SYBR14®	Green	Membrane permeant probe indicating live spermatozoa	[11, 14, 15]
	SYTOX® dyes	Various	Alternative group of nucleic acid stains with a mix of cell permeant and impairment properties with a variety of emission wavelengths. Used as an alternative to hoechst, PI, SYBR14® to reduce spectral overlap	[13, 16, 47, 89-90]
	7-AAD	Far red		[69]
	DRAQ® dyes	Far red		[36, 69]
Cytoplasmic	FDA, CFDA, CMFDA and CAM	Green	Cell permeant fluorescein based stains where viable cells are able to convert the stains to fluoresce with esterases and retained in the cytoplasm.	[13]
	Resazurin	Red	Redox indicator for cell viability assay where it is converted to red fluorescent resorufin in active cells	[86]
Cell membrane disorder	Merocyanine 540	Red or Far red	Lipophilic stain that binds to the outer leaflet of cell membranes of intact cells, it is internalized with membrane lipid disorder	[20-22]
Cell amines	Zombie®, Ghost® dyes	Various	Amine reactive dyes which are intensely fluorescent in membrane compromised cells where they enter the cell membrane and react with the intracellular free amines	[25]
Apoptotic markers	Annexin V	Conjugating fluorophore dependant	Cellular protein that binds to phosphotidylserine, on the outer leaf of the plasma membrane, often conjugated to fluorescein or Alexa fluor® dyes	[8, 18, 19, 23, 24]
	Yo-Pro-1, To-Pro-3	Green, Orange	Membrane impermeable probes, entering the cell only after membrane destabilization indicative of membrane damage, permeates into apoptotic cells	[17, 18, 88]
Acrosome integrity	FITC conjugated plant lectins	Green	*Arachis hypogaea* (peanut) agglutinin (PNA) and *Pisum sativum* (edible pea) agglutinin (PSA) binds to sugar moieties and on the outer acrosomal membrane	[8, 16, 22, 24, 26-31]
	FITC-conjugated immunoglobulins	Green	CD46 and other protein biomarkers have been identified for antibody specific assays for the acrosome reaction (human and mouse only)	[32 -34]
Mitochondrial	Rhodamine 123; JC-1, DiOC6(3), TMRM	Red; green to yellow/orange;	Mitochondrial membrane potential sensitive	[36-41]
	MitoTracker™ fluorochromes	Various	Cytosolic redox potential indicators	[38, 41]
Oxidative stress	H2CFDA	Green	Non-fluorescent and freely cell-permeable, until de-esterification intracellularly to become impermeant, non-fluorescent, H2DCF. In the presence of intracellular H2O2, it forms fluorescent 2′,7′-dichlorofluorescein (DCF)	[42-48]
	DHE; MITOSOX®	Deep red, far red	DHE is freely permeable non-fluorescent and is oxidized by superoxide and to a lesser extent other ROS, to ethidium, which binds to DNA and becomes fluorescent. MITOSOX® dyes are mitochondrion-targeted and a cationic derivative of DHE.	[36, 42,87]
	BODIPY® dyes, others	Red to green	Assessment of peroxidative damage due to lipid peroxidation.	[4, 42, 45, 48-52]
Intracellular calcium	Fluor dyes, Rhod-5N-AM	Various	Calcium ion indicators, often used assess calcium influx during capacitation	[8, 53-59]
Intracellular pH	BCECF, SNARF dyes, LysoSensor™ dyes	Various	pH dependent fluorescence intensities; indicates cell viability and functionality	[18, 22, 53, 60-61]
Chromatin	Acridine orange; FITC-dUTP	Red Green	SCSA assay and TUNEL assay; assesses defects to nuclear DNA (DNA fragmentation and breakage in single/double stranded DNA, respectively)	[73-76] [77]
	Chromomycin A_3	Dual: UV and far red	SPDA assay; used to assess sperm protamine integrity	[80-85]

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908 Table 2: Commercially available CASA systems with boar semen specific configurations.

CASA System name	Software	Vendor	Functions
AndroVision®	AndroVision® and AutoMorph	Minitube	Motility Concentration Morphology - unstained
ISAS v1	ISAS Lab®, ISAS PsUS, Trumorph®	PROiSER R+D	Motility Concentration Morphology – unstained and stained
SCA® platform	SCA® Motility and concentration; Morphology; Vitality DNA fragmentation Acrosome reaction Leukocytes	Microptic	Motility and concentration Morphology – stained Vitality DNA fragmentation Leukocytes
IVOS II™ and CEROS II™ analysers	Hamilton-Thorne with Boar Breeders II Software	Hamilton-Thorne	Motility Morphology

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