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Probes and Techniques for Sperm Evaluation by Flow Cytometry

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Contents

Flow cytometry has become an important technique in sperm evaluation and is increasingly used both for routine assessment and for research in veterinary science. We have revised the literature, describing fluorescent probes that have been used for analysing spermatozoa by flow cytometry, regarding: viability, acrosomal status, capacitation, mitochondrial status, apoptotic markers, oxidative stress markers, DNA damage, sperm counting and sperm sizing. Details and problems of some techniques are reviewed, with special attention to the occurrence of non-sperm particles in the samples ('debris'). New and promising aspects of flow cytometry, such as sperm sorting using viability markers as selection criteria, are highlighted. The relationship between flow cytometry analyses and fertility and their future improvements are considered.

Introduction

Flow cytometry has become widely used for sperm analysis, slowly replacing time-consuming and more error-prone techniques. Its ability for analysing multiple sperm characteristics at once makes it promising for new approaches in the analysis of sperm quality, which pursue the integration of different tests to achieve a better understanding of the spermatozoon functionality (Petrunkina et al. 2007). Moreover, sensitivity has increased, not only with the improvement of hardware, but also of fluorescence probes and labelling techniques.

Indeed, flow cytometers can be included in high throughput techniques, because they are able to analyse thousand of cells within seconds, capturing many features of each of them. Their repeatability is hardly reached by other techniques (a typical analysis of 10 000 spermatozoa yields a 95% C. I. <1%). In a flow cytometry analysis, labelled spermatozoa are driven within a laminar flow, passing one-by-one through a cell where they are illuminated by one or more lasers ('interrogation point'). The scattered or emitted light is filtered by mirrors and filters, reaching several photodetectors, where the signals are amplified. Finally, the information is digitalized and presented in different fluorescent intensity units to the researcher. Data is saved in standard FCS files (Flow Cytometry Standard). Thus, the information from each detector for each spermatozoon can be recovered and analysed afterwards. Typical sperm analyses usually render a few populations after manual classification. However, given the huge amounts of data that can be produced, some authors have proposed the use of complex statistical techniques to take advantage of the strengths of flow cytometry (Lo et al. 2008).

This review has a double objective. On the one hand, it is aimed at spermatologists with little experience in flow cytometry, giving them an overview of several sets of techniques already tested in spermatozoa. On the other hand, we want to present a thorough review of the literature to researchers already skilled in flow cytometry, suggesting new approaches. Nevertheless, the reader must keep in mind that the aim of flow cytometry is to achieve a deeper and more precise understanding of sperm physiology. Its ultimate purpose is to serve as a tool for the spermatologist, improving the diagnosis and prognosis in sperm samples and the evaluation of their fertility (Gillan et al. 2005).

Viability and Acrosomal Status

The membrane impermeable probes propidium iodide (PI) and ethidium homodimer (EH) are the most popular viability stains. They are easy to use, rapid and can be excited with the 488-nm laser that most cytometers include. These dyes enter cells with a broken plasmalemma, emitting red fluorescence when binding to nucleic acids (PI: 636 nm; EH: 617 nm) (Gillan et al. 2005). Other viability probes emit green fluorescence upon entering metabolically active cells (e.g. carboxyfluorescein diacetate) (Garner et al. 1986). The combination of PI and the SYBR-14 nucleic acid binding probe (Garner et al. 1994) is possibly the most widely used. SYBR-14 is membrane permeable, staining all sperm heads green. When PI penetrates in membrane-damaged spermatozoa, it displaces or quenches SYBR-14 fluorescence (Fig. 1a). Some authors have proposed 7-amino-actinomycin-D (7-AAD) as a viability marker (Perticarari et al. 2007), because it has an emission maxima at 647 nm (red), avoiding interference with green or orange dyes (spectral overlap). In a recent study, we employed a cyanine dye, TO-PRO®-3 dye (excited by a 635-nm He-Ne laser) for discriminating among viable and non-viable spermatozoa (Domínguez-Rebolledo et al. 2010). Fixable viability dyes are an emerging option (Perfetto et al. 2006). Fixable dyes are retained after fixation, allowing analysis to be delayed. Other advantage is the use of viability analysis together with techniques that require permeabilization (which may cause the loss of other viability dyes).

Acrosomal status has been assessed by using probes that recognize targets inside the acrosome, therefore labelling those spermatozoa with damaged or reacted acrosomes. The anti-CD46 antibody binds to the acrosomal matrix and has been used mainly in studies with human spermatozoa (Martin et al. 2005; Grunewald et al. 2006). Lectins, which bind to glucosidic residues in different parts of the acrosomal membrane, have been used with the same purpose in a wide range of

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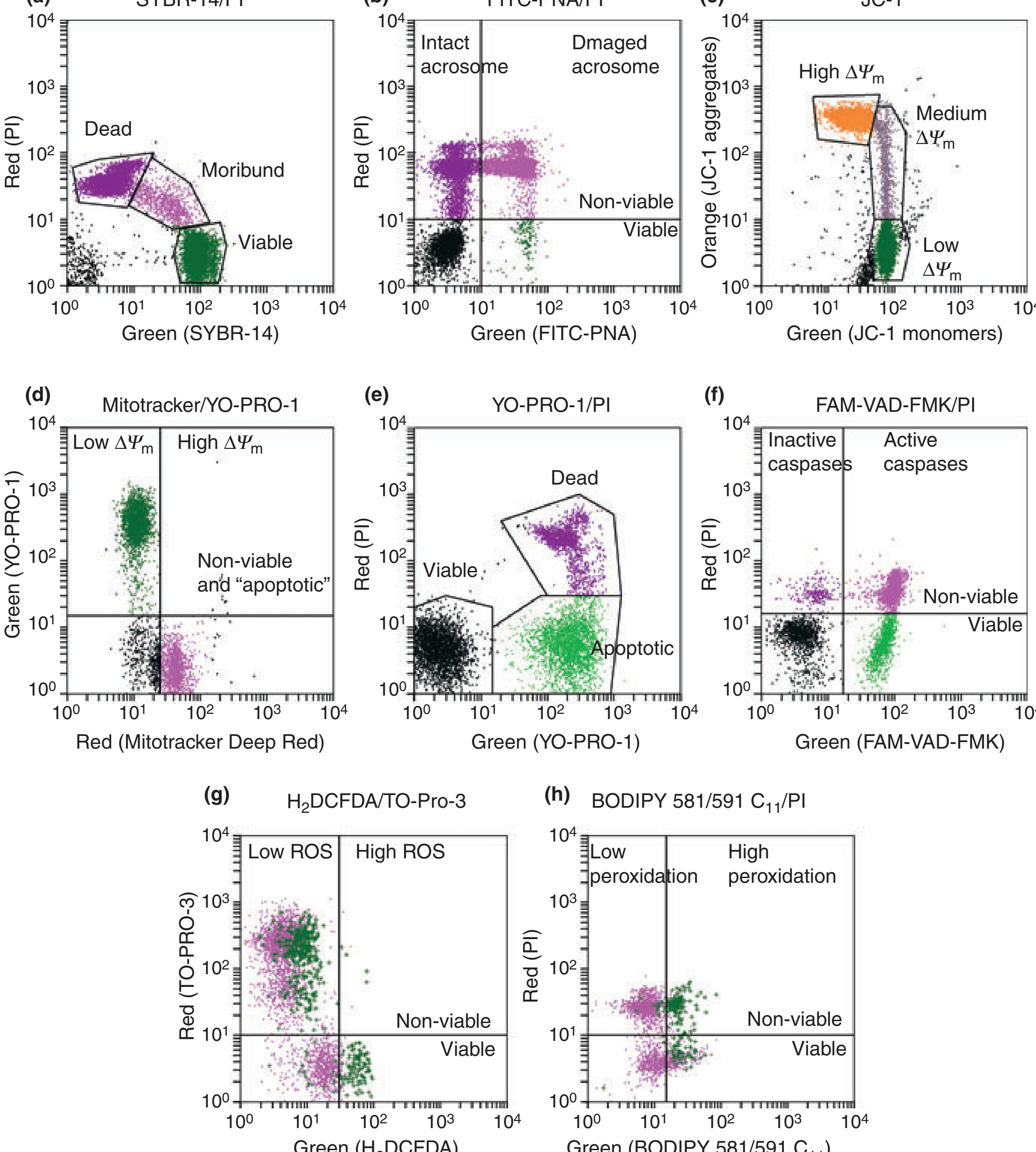

Fig. 1. Examples of flow cytometry analyses for assessing spermatozoa physiology. (a) Cytogram of a SYBR-14/PI stain. Three populations can be identified: dead spermatozoa (red stained), viable spermatozoa (green stained) and recently membrane-damaged, 'moribund', spermatozoa (red/green fluorescence). Unstained debris (events with low fluorescence) can be easily discarded. (b) Cytogram of a PI/FITC-PNA stain. Spermatozoa can be divided into intact/damaged acrosome and viable/non-viable, according to their green and red fluorescence. (c) Cytogram for the analysis of mitochondrial status using JC-1. Increasing orange fluorescence indicates higher $\triangle\psi_m$ (mitochondrial membrane potential); the high $\triangle\psi_m$ and medium $\triangle\psi_m$ are often difficult to separate. (d) Cytogram of spermatozoa stained with the viability dye YO-PRO®-1 and the mitochondrial probe MitoTracker® Deep Red FM. The double stain allows viable spermatozoa with or without active mitochondria to be discriminated. (e) cytogram of a YO-PRO®-1/PI stain. Whereas PI allows dead (membrane-damaged) spermatozoa to be discriminated, it cannot enter cells with increased membrane permeability, an apoptotic feature. YO-PRO®-1 labels such spermatozoa, separating those from the unstained non-apoptotic spermatozoa. (f) Cytogram obtained after incubating spermatozoa with the FAM-VAD-FMK fluorescent substrate and counterstaining with PI for viability. A fair proportion of membrane-intact spermatozoa (PI-) correspond to spermatozoa with activated caspases. A few spermatozoa were stained by PI while not showing caspase labelling, mostly corresponding to caspase-negative spermatozoa which were damaged during the washing steps of the protocol. (g) Overlay of two analyses after staining spermatozoa with the generic detector of oxidative species H_2DCFDA and the viability dye TO-PRO®-3, after incubating spermatozoa for 2 h at 37°C (dots), or incubating with 100 μM H_2O_2 (crosses). (h) Overlay of two analyses after staining spermatozoa with the lipid peroxidation marker BODIPY® 581/591 C_{11} and counterstained with the viability dye PI. Spermatozoa were incubated in buffered media for 6 h at 37°C (dots) or with 1 mM of *tert*-butyl hydroperoxide (crosses)

species. *Pisum sativum* (pea) agglutinin (PSA) (Tao et al. 1993) and *Arachis hypogaea* (peanut) agglutinin (PNA) (Tao et al. 1993; Thomas et al. 1997; Nagy et al. 2003; Martínez-Pastor et al. 2005) have been the most used lectins for that purpose, because of their specificity. Lectin- or antibody-labelled spermatozoa may be fixed,

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allowing for a later analysis if required. Acrosomal probes are generally conjugated with the green fluorochrome fluorescein isothiocyanate (FITC) and combined with the viability dyes PI or EH (Fig. 1b). Currently, they are available conjugated with fluorochromes emitting at different wavelengths. For instance, Nagy et al. (2003) proposed using PNA conjugated with the orange fluorochrome phycoerythrin (PE-PNA), which fitted between the green SYBR-14 and the red PI, and we have successfully used PNA conjugated with the orange fluorochrome tetramethylrhodamine isothiocyanate (TRITC), in a triple staining with YO-PRO®-1 and MitoTracker® Deep Red FM (Domínguez-Rebolledo et al. 2010).

Nevertheless, binding sites may disappear after extensive acrosomal damage, producing false negatives. Probes based on the physiological status of the acrosome, such as lysosomal probes (Thomas et al. 1997), might be more sensitive. Unfortunately, recent studies have been negative about the efficiency of acidic organelle probes, either because of heterogeneous labelling of the spermatozoa [LysoTracker® DND-99 and dapoxyl (2-aminoethyl) sulphonamide: Harper et al. (2006)] or because of unspecific labelling of the spermatozoa [LysoSensor™ Green DND-189: Castro-González et al. (2009)].

Evaluation of Sperm Capacitation Status

Spermatozoa capacitation is a key event for fertilization success. Thus, many fluorescence techniques have been adapted to flow cytometry, for measuring changes reporting capacitation. Chlortetracycline staining is the most representative technique developed for detecting capacitation in spermatozoa (Ward and Storey 1984), and Maxwell and Johnson (1997) reported having analysed it by flow cytometry. However, despite the interest in porting this technique to flow cytometry, no further studies have dealt with it. Merocyanine 540 (M540) has been used as an alternative for detecting capacitation (Harrison et al. 1996). This lipophilic probe binds to the plasmalemma, and its orange fluorescence (578 nm) increases with decreasing phospholipid membrane packaging, one of the events occurring during capacitation. However, its usefulness has been questioned, because M540 may detect cells with membrane degeneration as well (Muratori et al. 2004). M540 might still be useful for assessing membrane fluidity (Peña et al. 2004; Fernández-Santos et al. 2007; Caballero et al. 2009).

Capacitation involves cytosolic pH changes and increasing calcium levels. Thus, Pons-Rejraji et al. (2009) used the pH indicator 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF AM, emission at 535 nm) for measuring the capacitation-related pH increase in bovine spermatozoa. Indo-1 acetoxymethylester (Indo-1 AM) has been used for measuring intracellular Ca^{2+} in spermatozoa by flow cytometry (Brewis et al. 2000). The disadvantage of this probe is the need for a UV laser for excitation (e.g. 325 nm; emission at 475 nm without Ca^{2+} and at 401 nm with Ca^{2+}). An alternative is the fluo-3-acetomethoxy ester (Fluo-3 AM) probe, which is a green fluorochrome (526 nm) that can be excited by a conventional 488-nm laser, showing a large increase in fluorescence when bound to Ca^{2+} (Piehler et al. 2006; Caballero et al. 2009). Improved Ca^{2+} probes, such as Fluo-4 AM, could be adapted to flow cytometry in a similar way. Maturation, capacitation and other sperm traits are regulated by protein phosphorylation. Flow cytometry analysis of spermatozoa labelled with FITC-conjugated antibodies that recognize phosphotyrosines, either in the sperm surface (Piehler et al. 2006) or intracellularly (Barbonetti et al. 2008), has been used to detect such physiological changes.

Assessment of Mitochondrial Activity

Mitochondrial status is another important trait of sperm physiology (Peña et al. 2009). Rhodamine 123 was one of the first probes to be analysed with flow cytometry (Evenson et al. 1982), but this green fluorochrome has been displaced by improved ones, such as JC-1 or Mitotracker dyes (Garner et al. 1997). JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide) has been extensively used for sperm analysis. This probe accumulates in the mitochondria as a green-fluorescence monomer. In the presence of high mitochondrial membrane potential ($\triangle\psi_m$), the monomers form aggregates, shifting the fluorescence to orange. The flow cytometry analysis typically shows two or three populations (Fig. 1c), from events with high orange and low green fluorescence (high $\triangle\psi_m$) to events with low orange and high green fluorescence (low $\triangle\psi_m$). Several authors have used JC-1 with spermatozoa from different animal species [bull: Garner et al. (1997); ram: Martínez-Pastor et al. (2004); stallion: Ortega-Ferrusola et al. (2009)]. As a disadvantage, the double fluorescence of JC-1 may prevent this fluorochrome from combining with other probes in the green-red range. Furthermore, we have noted that JC-1 staining may be affected by many variables [as shown for brown bear semen by García-Macías et al. (2005)]. Mitochondrial probes, based on $\triangle\psi_m$, might be more sensitive to staining conditions than other probes, requiring more careful preparation and using controls for adjusting the cytometer.

$DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide) is another mitochondrial probe that has been used for analysing bovine semen (Martin et al. 2007). It increases its green fluorescence (501 nm) with high membrane potential. However, when Marchetti et al. (2004) compared $DiOC_6(3)$, TMRE (tetramethylrhodamine ethyl ester), CMX-Ros (Chloromethyl-X-rosamine) and JC-1, they found that only JC-1 was specific of mitochondria. Indeed, Brewis et al. (2000) used $DiOC_6(3)$ to study the plasma membrane potential, after depolarizing the mitochondria using the decoupling agent carbonyl cyanide m-chlorophenylhydrazone (CCCP).

MitoTracker® dyes are a group of recently developed dyes that accumulate and stain active mitochondria. As an advantage, they are highly specific, available in a wide range of emission fluorescence, and several of them are fixable, allowing combination with probes requiring fixation (e.g. antibodies) or delaying the analysis. Several of these probes have been used to evaluate

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spermatozoa in multicolour studies, such as MitoTracker® Green FM [bovine: Garner et al. (1997)], MitoTracker® Red FM [boar: (Gadella and Harrison 2002)], MitoTracker® Red CM-H_2TMRos [bovine: Fatehi et al. (2006)] and MitoTracker® Deep Red FM [stallion: Hallap et al. (2005); ram: García-Álvarez et al. (2009a,b); red deer: Domínguez-Rebolledo et al. (2009a); Martínez-Pastor et al. (2009a, 2008)]. MitoTracker® Deep Red FM (Fig. 1d) can be excited with a red laser (e.g. a 635-nm He-Ne laser), allowing it to be combined with fluorochromes excited with the blue laser (488-nm line), therefore achieving a lower risk of fluorescence spill-over.

Detection of Apoptosis-Like Changes

Apoptosis-like changes have been detected in spermatozoa, and the use of flow cytometry has greatly helped its study (Pena 2007). One of the early steps of apoptosis is the translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, which can be detected by annexin V labelling. This marker, conjugated with FITC, has been analysed by flow cytometry to detect subtle membrane changes related to sperm processing, freezing/thawing or other stresses in several domestic species [bull: Anzar et al. (2002), Januskauskas et al. (2003); boar: Gadella and Harrison (2002), Petrunkina et al. (2005), Peña et al. (2005a)]. Currently, annexin V is available conjugated with a wide variety of fluorochromes, allowing it to be combined with a wider range of probes.

Increased membrane permeability is another indicator of apoptosis. YO-PRO®-1 iodide is a green (509 nm) cyanine dye that is able to stain the nucleus of apoptotic cells (Peña et al. 2005b). This dye has been combined with PI or EH (Fig. 1e) to identify membrane-undamaged spermatozoa with increased membrane permeability, for instance, in bull (Januskauskas et al. 2005; Hallap et al. 2006), stallion (Núñez-Martínez et al. 2007), boar (Peña et al. 2005b, 2007; Kumaresan et al. 2009), ram (García-Álvarez et al. 2009a,b) or red deer (Martínez-Pastor et al. 2008, 2009a; Domínguez-Rebolledo et al. 2009a). YO-PRO®-1 has been combined with other probes, such as merocyanine 540, PNA-TRITC or MitoTracker® Deep Red FM, to take advantage of its high sensitivity to discriminate membrane-intact spermatozoa (Peña et al. 2004; Caballero et al. 2009; García-Álvarez et al. 2009b; Martínez-Pastor et al. 2009a; Domínguez-Rebolledo et al. 2010). Other dyes have shown to be specific of either intact, non-apoptotic spermatozoa [carboxy SNARF®-1 AM (Peña et al. 2005b)], or of spermatozoa with apoptotic features [SYTO® 16 (Perticarari et al. 2007)].

Caspases are the central components in the apoptosis signalling cascade. Members of this family of cysteine proteases have been found in their inactive and active state in spermatozoa (Grunewald et al. 2009). Membrane-permeable fluorescent caspase inhibitors (FLICA™) have been used to detect caspase activity in several cell types. The FLICA™ reagent is composed of a carboxyfluorescein (green) reporter group (FAM), an aminoacid peptide inhibitor sequence that acts as the recognition site, and a fluoromethyl ketone moiety (FMK) that reacts covalently with the cysteine of the reactive centre. The pan-caspase or poly-caspase substrate FAM-VAD-FMK (VAD: valine-alanine-aspartic acid) is the most frequently reported for detecting activated caspases in spermatozoa by flow cytometry (Fig. 1f). This assay has been used with flow cytometry in samples from boar (Moran et al. 2008), red deer (Martínez-Pastor et al. 2009a) and stallion (Ortega-Ferrusola et al. 2009). Some authors have investigated the presence of specific caspases, especially caspase 3 (using the FAM-DEVD-FMK reactive) (Paasch et al. 2004; Grunewald et al. 2008b). Martí et al. (2008) successfully detected caspase 3 and caspase 7 in ram spermatozoa, although they did not test the technique with flow cytometry. Interestingly, Grunewald et al. (2008a) showed that the caspase labelling (caspase-3 FLICA™) was stable enough to evaluate sperm samples up to 10 days after staining and fixing with 4% paraformaldehyde, which could be suitable for delayed analysis.

Other markers have been explored, as reporters of abortive apoptosis during spermatogenesis or defective sperm maturation. It is known that abnormal spermatozoa are tagged by ubiquitination of the plasma membrane in the testis and epididymis (Sutovsky et al. 2003). These spermatozoa may appear in the ejaculate, indicating infertility or poor semen quality. Sperm ubiquitination can be detected by immunofluorescence (sperm ubiquitin tag immunoassay, SUTI) and flow cytometry (Sutovsky et al. 2003; Purdy 2008).

Detection of Oxidative Stress

Oxidant species have a crucial role in sperm physiology, but they may impair the fertilizing ability of the spermatozoa. Therefore, their detection and the evaluation of oxidative stress are important both for physiological and for pathological studies. The detection of reactive oxygen species (ROS) and other oxidative species can be carried out by reagents that accumulate intracellularly and become fluorescent upon oxidation. The 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) has been used as a generic reporter of intracellular ROS in spermatozoa [e.g. in dog (Kim et al. 2010), red deer (Martínez-Pastor et al. 2009a; Domínguez-Rebolledo et al. 2010)]. H_2DCFDA penetrates the plasma membrane, it is retained after intracellular esterases cleave the acetate groups and emits green fluorescent (504 nm) upon oxidization. CM-H_2DCFDA (5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester), which we tested recently in red deer spermatozoa (Domínguez-Rebolledo et al. 2010), is better retained within live cells. These probes can be combined with viability dyes for removing the non-viable population from the analysis (Fig. 1g). Whereas H_2DCFDA is non-specific, some probes may detect specific oxidant species. Dihydroethidium (DHE) reacts with the superoxide anion (O_2^-), forming the yellow (567 nm) 2-hydroxyethidium (Zhao et al. 2005). However, most studies have reported using red fluorescence filters for detecting the signal from the products of oxidized DHE (Burnaugh et al. 2007; Koppers et al. 2008), therefore confounding the oxidation of DHE to

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ethidium (E^+; red fluorescence at 617 nm) for oxidant species other than $O_2^{-\cdot}$. Furthermore, the detection of DHE products by fluorescence alone has been discouraged (Zielonka et al. 2009). The MitoSOX™ Red may be a more adequate option for selectively detecting $O_2^{-\cdot}$ (Koppers et al. 2008). The presence of nitric oxide ($NO^\cdot$), an important short-lived messenger and potential generator of peroxynitrite, has been assessed by flow cytometry in boar and stallion spermatozoa (Moran et al. 2008; Ortega Ferrusola et al. 2009a) by using 4,5-diaminofluorescein diacetate (DAF-2 DA). Currently, a more sensitive and stable probe is available for that purpose (DAF-FM diacetate).

Oxidant attack on membranes results in lipid peroxidation, negatively affecting sperm quality. BODIPY® 581/591 C_{11} has been used to evaluate lipid peroxidation using flow cytometry analysis of spermatozoa [bull: (Brouwers and Gadella 2003); red deer: (Domínguez-Rebolledo et al. 2009b); stallion: (Ortega Ferrusola et al. 2009b)]. This probe emits orange fluorescence in its non-oxidized state (excitation at 581 nm and emission 591 nm), shifting to green fluorescence when peroxidized (excitation at 500 nm and emission at 510 nm). Thus, it is possible to perform a ratiometric analysis (Christova et al. 2004), but generally only the green fluorescence is reported in flow cytometry studies. This probe can be combined with viability stains to discard non-viable spermatozoa (Fig. 1h). BODIPY® 665/676 is a similar probe emitting red fluorescence, which we have tested in red deer spermatozoa (Martínez-Pastor et al. 2009a), finding similar results when comparing to BODIPY® 581/591 C_{11} (Domínguez-Rebolledo et al. 2009b).

Other probes could be used to assess oxidative stress in spermatozoa by flow cytometry. For instance, 5-iodoacetamidofluoresceine (5-IAF), a green probe for detecting reduced sulfhydryl groups on the sperm surface, has been used for detecting thiol oxidation in boar (Gadea et al. 2005). Similar to iodoacetamides, maleimides are potentially useful probes. They are more specific for reduced thiols and available in a broad range of fluorescence.

Assessment of Chromatin Status

Damaged sperm chromatin may impair the capability of the spermatozoa to fertilize, decrease IVF or insemination success, cause abortion or foetal abnormalities and even reduce offspring fitness (Virro et al. 2004). The SCSA® (sperm chromatin stability assay) is the most widespread test for assessing sperm chromatin by flow cytometry. This test is relatively simple to perform: sperm samples are submitted to a DNA denaturation step, mixed with an acridine orange (AO) solution and analysed by flow cytometry (Evenson and Jost 2000). AO is a metachromatic fluorochrome that readily intercalates in the DNA. When associated to double-stranded DNA (dsDNA) it fluoresces green, whereas it fluoresces red when associated to single-stranded DNA (ssDNA). The denaturation step induces the formation of ssDNA from breakages, therefore each sperm head yield a mixture of green and red fluorescence when interrogated with a 488-nm laser, depending on the DNA fragmentation (number of nicks) and the susceptibility of chromatin to denaturation (Fig. 2). Data is processed to obtain, for each spermatozoon, the red/[total fluorescence] ratio, called the DNA fragmentation index (DFI). A series of cut-off values can be set for DFI, to obtain the percentage of spermatozoa with moderate DFI (cut-off at 0.25 DFI) and with high DFI (cut-off at 0.75 DFI). It is important to consider that the DFI acronym is used both to refer to the red/total fluorescence ratio of individual spermatozoa (formerly termed α_t) and to refer to the percentage of spermatozoa

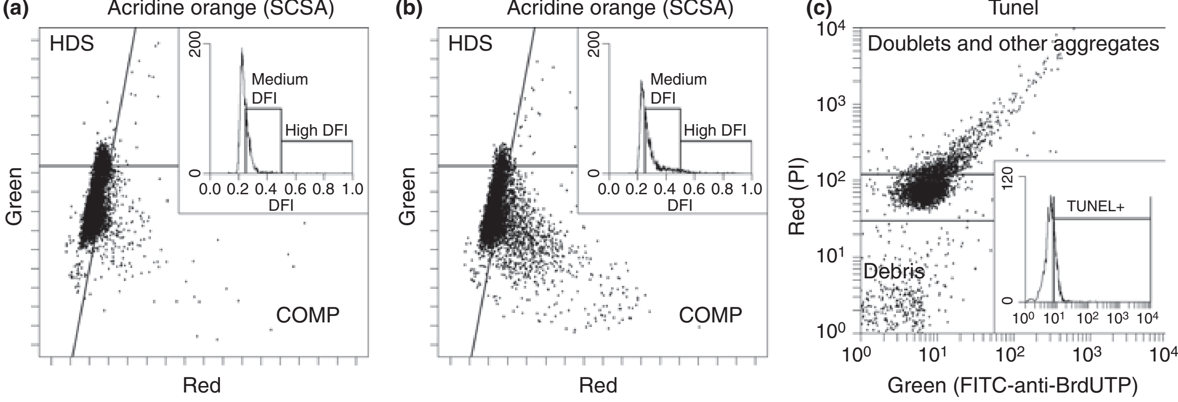

Fig. 2. Assessment of sperm chromatin. Cytograms (a) and (b) were obtained after carrying out the SCSA® protocol. Acridine orange yields different amounts of red and green fluorescence depending on the dsDNA and ssDNA present in the nucleus. Cytogram (a) correspond to a sample with few spermatozoa showing medium or high DFI (red fluorescence/total fluorescence). Cells plotted to the right of the diagonal line have increased DFI, also termed 'COMP' (cells out of the main population). The inset shows the histogram of DFI, with most events within the low-DFI peak. Conversely, the cytogram (b) shows a higher number of spermatozoa in the COMP region, because of increasing DFI levels (as shown in the inset). Debris (events with low green fluorescence and in the low-green/low-red region) was previously removed from the analysis. Cytogram (c) shows spermatozoa treated with the TUNEL protocol and counterstained with PI. Note that PI allows debris (events with low red fluorescence) and aggregated spermatozoa (events with increasing red fluorescence above the main sperm population) to be removed. Green fluorescence, in this case because of an anti-BrdUTP antibody conjugated to FITC, increases with the occurrence of DNA single strand breaks and can be easily analysed using a histogram of the distribution of green fluorescence (inset), after gating out debris and aggregates, and setting the TUNEL+ marker by using positive and negative controls

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with moderate and high DFI (formerly termed COM-$P\alpha_t$). Evenson et al. (2002) also defined high DNA stainability [HDS, formerly termed 'HIGRN' (Evenson and Jost 2000)] as a measure of the condensation degree of the sperm chromatin, and possibly related to chromatin alterations and infertility (Virro et al. 2004). A high DFI has been related to reduced fertility, longer times to pregnancy and higher spontaneous miscarriage rates in humans (Virro et al. 2004; Evenson and Wixon 2006), and it has shown a relation with fertility and prolificacy in domestic animals [bull: Waterhouse et al. (2006), García-Macías et al. (2007); boar: Boe-Hansen et al. (2008)]. We have used the SCSA® in ram, Iberian red deer, bull, domestic dog and bear, finding it sensitive for detecting seasonal changes in chromatin condensation (García-Macías et al. 2006a), comparing epididymal and ejaculated spermatozoa (García-Macías et al. 2006b) and assessing the effect of refrigeration or cryopreservation on spermatozoa (Martínez-Pastor et al. 2004, 2009b; Fernández-Santos et al. 2009a,b,c). A caveat to researchers implementing this test is to carefully follow the developers' guidelines (Evenson and Jost 2000; Evenson et al. 2002). Although the SCSA® is relatively easy to follow, many factors can influence its results (Boe-Hansen et al. 2005). Thus, the preparation of a standard sample, denaturation and staining conditions and times must be strictly controlled, and the AO must be of the highest quality (chromatographically purified). Adjustment of the sperm concentration and dilution with the AO solution must be carefully performed, because AO must be at equilibrium with the sperm sample (regarding the molar relationship among AO and base pairs).

Another DNA assay that has been adapted to flow cytometry is the TUNEL assay (terminal transferase dUTP nick end labelling). Originally devised to detect DNA degradation in apoptotic somatic cells, it uses a terminal deoxynucleotidyl transferase to add deoxyuridine triphosphate nucleotides to the 3′-hydroxyl ends resulting from DNA breaks. The 3′-hydroxyl ends are labelled either by using fluorescent-tagged dUTP or by using 5-bromo-dUTP and incubating the marked sample with an anti-BrdUTP antibody conjugated with a fluorochrome (Fig. 2c). This assay is more expensive and more complex to perform than the SCSA®, but it provides precise information about the degree of sperm fragmentation (because fluorescence increases with the number of 3′-hydroxyl ends). Thus, the TUNEL is a promising technique that has demonstrated a good relationship with SCSA® and with fertility (Waterhouse et al. 2006; Benchaib et al. 2007). We have shown that the TUNEL assay combined with flow cytometry could discriminate among different sperm treatments that could cause an increase in DNA breaks in red deer spermatozoa (Domínguez-Rebolledo et al. 2009a, 2010). We have used PI to remove debris and sperm aggregates, better defining the sperm population (Fig. 2c). As other authors reported previously (Muratori et al. 2008), we have noticed changes in the PI fluorescence intensity of spermatozoa when high DNA damage was present. We must highlight the need of employing negative and positive controls, in a similar manner than for SCSA®. The positive control can be easily made by incubating the spermatozoa with DNase I. Moreover, washing steps can greatly decrease the sperm concentration if not carried out carefully, causing artefacts when analysing the samples.

Recently, other methods have been adapted for flow cytometry. Chromatin thiol status and compaction may be assessed by using monobromobimane (mBBr) and chromomycin A3 (CMA3), respectively (Zubkova et al. 2005). A high degree of DNA compaction in the sperm head limits the accessibility of DNA-damaging agents and depends on the number of disulphide bonds within and between protamine molecules. Faulty spermatogenesis or maturation can impair chromatin compaction. Thus, Zubkova et al. (2005) found a decrease in chromatin compaction (CMA3) with age in rat sperm and an increase in thiol oxidation after oxidative stress. Therefore, these probes could be useful in the quality assessment of semen doses from domestic animals.

Thus, the TUNEL is a promising technique that has shown a good relationship with SCSA® and with fertility (Waterhouse et al. 2006; Benchaib et al. 2007).

Using the Flow Cytometer for Sperm Counting and Sizing

Because a flow cytometer can acquire thousands of events in several seconds at a fixed flow rate, it could be used for assessing sperm concentration. However, cytometers must deal with electronic noise, unstable flow and other factors, which may disable them as accurate counting devices (Lu et al. 2007). Nevertheless, some authors have obtained good results when comparing with well-established methods (Prathalingam et al. 2006). Good flow adjustment and the presence of internal controls (latex beads or fluorospheres at standard concentrations) can improve the reliability of concentration measurements (Eustache et al. 2001).

Sperm sizing could be also performed by flow cytometry, in theory. The FSC (forward scatter of the laser light) has been traditionally considered proportional to the size of the event intercepting the laser. In practise, the FSC as a measure of particle size is unreliable (Shapiro 2003). FSC and SSC could still be used to study spermatozoa morphological changes (Martínez-Pastor et al. 2008), but extrapolating such data to real spermatozoa morphology would be foolhardy. In fact, the only automatic devices which can accurately assess spermatozoa volume are those based on the Coulter effect Petrunkina et al. (2004). However, Coulter-based devices lack the fluorescence detection capabilities of regular flow cytometers. Currently, there is a commercial cytometer that makes use of the Coulter effect (Cell Lab Quanta™; Beckman Coulter), which has been used for simultaneously studying cell volume changes while utilizing fluorescent probes (Bortner et al. 2007). Nevertheless, no studies have been reported on spermatozoa up to date.

Removing Non-Sperm Events (Debris)

One of the main problems of analysing spermatozoa by flow cytometry is the presence of non-sperm events in the sample stream: bacteria, blood cells, epithelial cells,

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tissue, gel particles or extender particles (e.g. egg yolk). These events or 'debris' may be discarded by gating in a FSC/SSC dot plot (based on differences in 'size' and 'complexity' among debris and spermatozoa), but this is not always possible. Furthermore, debris may show autofluorescence, overlapping with sperm populations in fluorescence plots. Even worse, debris may be labelled by some probes (for instance, egg yolk particles are strongly stained with lipophilic probes, such as merocyanine 540). In that case, one or more sperm populations will be overestimated. If the interference is considerable and there is no way to remove it, the validity of the flow cytometer analysis in that particular experiment should be reconsidered. Recently, Petrunkina and Harrison (2009) studied the misestimation of sperm populations because of debris, proposing a series of corrective measures by mathematically post-processing flow cytometry results, which may be useful in some cases. Nagy et al. (2003) proposed an ingenious method for separating spermatozoa from debris in 'dirty' samples using the double stain SYBR-14/PI; because these probes only label nucleated events, debris stay unstained (Fig. 1a). Other authors suggested using Hoechst 33342 (Fig. 3), which labels nuclei with blue fluorescence, thus leaving the green–red photodetectors available for other probes (SYBR-14/PI require the green and red photodetectors). An important drawback of Hoechst probes is that the cytometer must be equipped with an ultraviolet laser (which increases the cost of the device). Nevertheless, Hoechst 33342 can be also excited with a solid-state violet laser (Alcaide et al. 2009). We have tested both SYBR-14/PI and Hoechst 33342, and results have been from good to optimal discriminating among spermatozoa and debris when combining FSC/SSC and fluorescence plots (Martínez-Pastor et al. 2008, 2009a; Castro-González et al. 2009; Domínguez-Rebolledo et al. 2009a; García-Álvarez et al. 2009a,b).

Sperm Sorting

It is indubitable that sperm sex sorting, the ability of separating X and Y spermatozoa by minute differences in DNA content, has been one of the greatest practical applications of flow cytometry to the field of assisted reproduction. Sex sorting requires specialized cytometers and standardized protocols regarding staining, flow and media. This topic is rich and complex enough to escape this review; therefore, the reader is referred to other studies (Garner and Seidel 2008; de Graaf et al. 2009; Sharpe and Evans 2009; Vazquez et al. 2009). Nevertheless, conventional cell sorters, such as MoFlo™ (Beckman Coulter, Brea, CA, USA) or FACSAria™ (Becton Dickinson, Franklin Lakes, NJ, USA), could be used for sperm sorting, based on viability, apoptotic or other markers. Hoogendijk et al. (2009) have reported using a FACScalibur™ flow cytometer (Becton Dickinson) equipped with a FACSort™ fluidic sorting module 11 for sorting human spermatozoa using as criteria annexin V-FITC labelling. The possibility of physically sorting a sperm population depending on one or several parameters opens new possibilities for research and for practical use.

Flow Cytometry Analysis of Spermatozoa and Fertility Assessment

The search for a single analysis that could predict fertility has been mostly fruitless. Many *in vitro* assays have shown correlations with fertility, but in most cases they have been highly variable depending on the study, and only the combination of several tests have led to high correlations (Rodriguez-Martinez 2003). The use of flow cytometry, despite its high sensitivity and repeatability, has not improved this situation, except in the case of DNA analysis by SCSA®. As indicated earlier, many authors have reported that field fertility in bull and boar correlate with SCSA® results, and some threshold limits for DFI have been proposed, to identify subfertile individuals (Waterhouse et al. 2006; Boe-Hansen et al. 2008; Didion et al. 2009). Nevertheless, most studies have found that samples from domestic cattle usually yield DFI within a narrow range, reducing the predictive value of this test for fertile animals.

Flow cytometry analyses are that detect subtle changes in the sperm physiology could be associated

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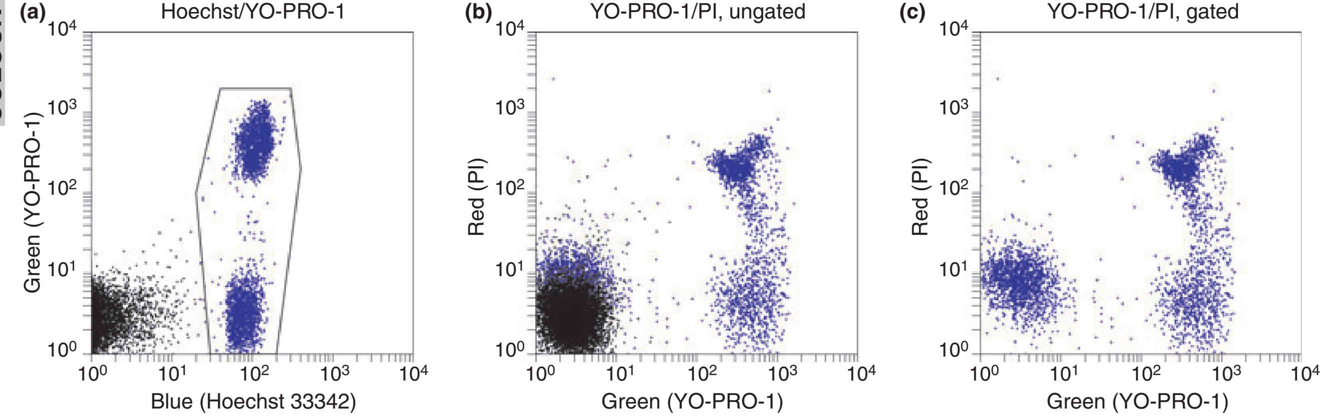

Fig. 3. An example of the use of Hoechst 33342 for removing debris from a YO-PRO-1/PI analysis (see Fig. 1e). Cytogram (a) shows how nucleated events (high blue fluorescence) are well separated from debris (low blue fluorescence) and can be gated by drawing a region in the dot plot. Debris is evident in the ungated YO-PRO-1/PI cytogram (b), inflating the unstained (viable) population. After removing the debris, the result of the analysis changes considerably (c)

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to the fertility of spermatozoa. For instance, Anzar et al. (2002) showed good correlations between the results of the annexin V/PI and TUNEL assays of fresh semen with fertility results in cattle. However, correlations dropped to non-significance when analysing thawed semen. Moreover, Januskauskas et al. (2003) found low correlations (r slightly above −0.5) among flow cytometry parameters and fertility (COMPα_t parameter of the SCSA and the annexin V−/PI+ population of the phosphatidyl serine externalization assay). Only after combining flow cytometry with other assays (fluorometry and CASA) did these authors obtain high correlations ($r = 0.9$). Therefore, some flow cytometry analyses present a promising prospect for evaluating the fertility potential of semen samples, but results so far show a high dependence of experimental conditions, and a reduced power when assessing samples of selected animals (e.g. AI-bulls) (Rodriguez-Martinez 2006).

Rather than aiming at finding a single relationship between fertility and the result of semen analyses, new strategies tend to combine functional tests, which challenge spermatozoa, with sperm analyses. These tests allow the true fertility potential to be unveiled, taking advantage of the natural heterogeneity of sperm samples (Rodriguez-Martinez 2006; Petrunkina et al. 2007). For instance, Bollwein et al. (2008) obtained no relationship between SCSA® and field fertility working with bull semen. However, after incubating thawed samples at 38°C for 3 h they obtained a negative correlation of DFI with the 56-days non-return rate. The development of evaluation tests combining physiological challenges with advanced analytical techniques, such as flow cytometry, is expected to improve the predictive value of *in vitro* assessment with relation to fertility.

Future Perspectives

In this review, we have presented several techniques that have been recently used for sperm analysis by flow cytometry, or that could be readily adapted for this cell type. Nevertheless, other advances in the field of flow cytometry could be extremely useful for studies on spermatology, and they must be kept in mind when starting new research projects. For instance, we have described several techniques based on immunostaining (e.g. detection of tyrosine phosphorylation or sperm ubiquitination). The number of possible targets related to sperm quality is increasing, and it is possible that some of them could enable sperm analysis for predicting freezability and fertility to be improved (Gillan et al. 2005; Love 2005; Evenson and Wixon 2006). An increasing number of techniques are expected to be adapted to flow cytometry analysis, thus allowing for high throughput, cell-to-cell analysis of these markers. Simultaneously, multicolour cytometry of spermatozoa must be refined to allow for multiple detection in a single analysis. Advanced analytical techniques, such as multiparametric clustering, might be necessary (Lo et al. 2008).

Cytometry imaging has filled the gap among flow cytometry and fluorescent microscopy. A disadvantage of flow cytometry is the impossibility of locating the fluorescence on the spermatozoa. In cytometry imaging, the flow cytometer is provided with charge-coupled device (CCD) cameras instead of the typical photodetectors, allowing images of the cells to be obtained while they cross the laser beam. The possibilities of these new cytometers for spermatology are endless (Buckman et al. 2009). A different technology, the silico-image incorporated in CytoSense instruments (CytoBuoy, Woerden, the Netherlands), performs length scans of the analysed particles as they pass through the laser. Therefore, each particle is characterized not only by a single fluorescent intensity per photodetector, but also by fluorescence profiles. Taking into account the morphology of the spermatozoa and its high degree of compartmentalization, combining morphometric analysis, fluorescence detection and fluorescence localization could boost some research areas.

Future improvements and achievements on flow cytometry must be linked to results from functional tests and *in vitro* and field fertility. Ultimately, the usefulness of these developments should result in better prediction of sperm fertility and in the practical application to enhance the results of assisted reproductive techniques.

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Conflict of Interest

Xxxxx. **12**

Author contributions

Xxxxx. **13**

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