https://doi.org/10.17221/152/2023-CJAS

Meta-analysis of fluorochrome concentrations to assess bull sperm quality by flow cytometry in 2021–2023

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Citation: Savvulidi F.G., Ptáček M., Nagy S., Stádník L. (2023): Meta-analysis of fluorochrome concentrations to assess bull sperm quality by flow cytometry in 2021–2023. Czech J. Anim. Sci., 68: 469–485.

Abstract: Flow cytometry (FC) is a very powerful method for the assessment of spermatozoa quality in multiple livestock species, including bulls. Nowadays, many flow cytometric tests are available for quality detection of bull sperm. These tests are based on fluorochromes (fluorescent tags or dyes) and the use of correct concentrations of fluorochromes is an essential aspect of the successful optimization of experimental methodology. Modern flow cytometers are much more affordable, such that spermatologists are now able to use FC methodology (and even image cytometry) in their studies. The present review aims to give a summary of current flow cytometric methodologies used to assess bull sperm quality by flow cytometry. Namely, we give here the concentrations of fluorochromes used in the studies related to bull sperm quality evaluation by flow cytometry published in the last three years (from 2021 to 2023). Importantly, in the present review, the concentration of fluorochromes is unified and presented using comparable molarity units. Furthermore, the rationale of each flow cytometric methodology for particular fluorescent tests, and the citation for the corresponding original methodology, are given. This review will help spermatologists shorten the time needed for the optimization of their flow cytometry methodology for the assessment of bull sperm quality.

Keywords: DNA stability; plasma membrane integrity; acrosomal status; mitochondrial status; oxidative stress; intracellular calcium; analysis accuracy; bovine sperm

There are currently a number of available in vitro assays for the evaluation of bull sperm quality with the use of flow cytometry. A plethora of important quantitative characteristics of sperm cells can be assessed through flow cytometry. For example, the level of DNA protamination, chromatin compaction, and susceptibility of DNA to fragmentation, especially considering the integrity of spermatozoa plasma membrane, the sperm acrosomal and mitochondrial status, the level of sperm oxidative stress, or the evaluation of intracellular calcium are all the essential important qualitative parameters. With the present review we are not aiming to give a detailed overview of the quantitative characteristics of spermatozoa which might/should be assessed; such information should be searched elsewhere (Amann and Hammerstedt 1993; Muller 2000; Cordelli et al. 2005; Petrunkina

Supported by the Project NAZV QK22010270 and by CULS SGS Grant No. SV23-7-21320.

https://doi.org/10.17221/152/2023-CJAS

et al. 2007; Sigman et al. 2009; Martinez-Pastor et al. 2010; Hossain et al. 2011; Sellem et al. 2015; Gliozzi et al. 2017; Kumaresan et al. 2017; Ortega-Ferrusola et al. 2017; Vasicek et al. 2022; Nagy et al. 2023; Pena 2023). Instead, this review is intended for spermatologists who already know what qualitative parameters they are going to assess by flow cytometry, such as the DNA stability, the integrity of plasma membrane, the acrosomal and mitochondrial status, the level of oxidative stress, the level of intracellular calcium. In addition, this review may serve as a tool for those who may be searching for the information, particularly about the fluorescent dye concentration to use. In general, when preparing a flow cytometric measurement of sperm cells, preliminary titration of fluorochromes to find their optimal concentrations is an important consideration. However, this stage is often ignored mainly due to the significant time costs associated with titration. When preparing a cytometric measurement, spermatologists prefer to find already established and published dye concentrations. Therefore, our task in writing this review was to describe the concentrations of fluorochromes and to facilitate the search for our colleagues working in areas where flow cytometric analysis of bull sperm quality is needed. We believe that our review will be useful to spermatologists in determining the approximate magnitude of concentrations for the described tags. This can, to some extent, replace the need for the dye titration process. On the other hand, we stress here the importance of the titration procedure to find dye concentration that is optimal for particular experimental settings. We consider it necessary to emphasize that the discussion of the correlation between the results of parametric analysis of bull sperm using flow cytometry and the fertilization ability of the semen is beyond the scope of this review. The same applies to the flow cytometric gating strategy: we recommend readers find particular information on the gating strategy (with illustrative dot-plots of fluorescently stained spermatozoa with fluorochromes) elsewhere. Several excellent studies can be recommended on this issue (Martinez-Pastor et al. 2010; Hossain et al. 2011; Vasicek et al. 2022).

The present review aims to give a summary of current flow cytometric methodologies used to assess bull sperm quality by flow cytometry. Particularly, we aim to give the approximate magnitudes of concentrations for the described fluorochromes. Because we present here fluorescent dye concentrations in a comparable manner, this review should help spermatologists shorten the time needed for the optimization of their flow cytometry methodology for the assessment of bull sperm quality.

COMPREHENSIVE REVIEW OF METHODS

To search for articles on the topic of our review, we used the search engine PubMed (https://pubmed.ncbi.nlm.nih.gov/) with the following keywords: flow cytometry, bull sperm, bovine sperm, sperm quality. The search results were limited to only original scientific articles published within the last three years (2021–2023). In our review, we tried to keep an original methodology description, to prevent introducing of mistakes or mistypes when describing the methodology. Therefore, in the present review readers might observe some heterogeneity between methodologies employed by different authors. When possible, the original protocol paper (even if it was published before 2021) is cited together with its corresponding article published within the last three years. Also, when possible, the laser light source and laser power are given. Below are the search results in the form of brief methodological approaches, summarized in the following groups:

Evaluation of sperm chromatin condensation and DNA stability

It is possible to detect the amount of protamine by the use of the fluorescent dye Chromomycin A3 (CMA3) with a flow cytometric assay. Alfadel et al. (2023) used the CMA3 staining methodology by Simoes et al. (2009), but replaced the fluorescent microscopy with flow cytometry. Briefly, thawed sperm samples were fixed for 5 min in Carnoy's solution (3 : 1, methanol : glacial acetic acid) at 4 °C. Fixed samples were washed in phosphate-buffered saline (PBS) (Ca^{+2} and Mg^{+2} free) and centrifuged. Pellets were stained with a CMA3 solution (0.25 mg/ml) for 20 min in the dark at room temperature. Samples were analysed using a flow cytometer BD FACSCANTO™ II. The 583-nm detector was used for protamine deficiency. A positive control for CMA3 staining was prepared using sperma-

https://doi.org/10.17221/152/2023-CJAS

tozoa from a random bull that underwent sperm nucleus deprotamination according to Simoes et al. (2009). Another authors' collective (Ribas-Maynou et al. 2022a) described an additional methodology where samples at a concentration of 20 × 10^6 sperm/ml were diluted 1 : 1 (v : v) in 2X McIlvine solution (60 mmol/l citric acid, 280 mmol/l Na_2HPO_4 and 20 mmol/l $MgCl_2$) containing 12.5 µg/ml CMA3, and incubated for 30 min at room temperature. Subsequently, samples were diluted 1 : 10 (v : v) in PBS, and examined with a CytoFlex flow cytometer (Beckman Coulter, Fullerton, CA, USA), equipped with red, blue, and violet lasers (637, 488 and 405 nm). Chromomycin (CMA3) was excited with the 405-nm laser and its fluorescence was collected with the Violet610 channel (610/20 nm). The fluorescence intensity for Violet610 channel was recorded as a quantitative measure of deprotamination degree. A negative control without CMA3 was conducted in parallel to establish the threshold value for positive CMA3 cells, leading to the final identification of two sperm populations: CMA3+ and CMA3–. The percentage of sperm with a high degree of deprotamination (CMA3+) was recorded.

Chromatin thiol status (the number of disulphide bonds) in bull spermatozoa was recently assessed by Salman et al. (2023) with the analysis of free thiols and estimation of disulphide bridges performed with the monobromobimane (mBBr). Samples were prepared in tris-HCl, NaCl, ethylenediaminetetraacetic acid (EDTA) buffer solution (TNE) at 1 × 10^6 spz/ml and pipetted in parallel black 96-well plates. One of the plates was used as a reference by reducing the disulphide bridges to free thiols with a 1 mM DTT treatment (dithiothreitol; 10 min, 37 °C). The reference plate was washed and resuspended, and both plates were processed in parallel. The mBBr fluorochrome was added at 500 µM in PBS and incubated for 10 min at 37 °C. After washing with PBS, the samples were stained with propidium iodide and left at 4 °C in the dark for 24 h. The plates were analysed in a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotech, Bergisch Gladbach, Germany), reading 10 × 10^3 spermatozoa per sample. The mBBr was excited with violet line (405 nm), and its emission was detected using a 450/50 nm filter. Disulphide bridges were estimated by subtracting the mean fluorescent intensity (MFI) of the samples from the reference plate (DTT-treated) and dividing by two (Zubkova et al. 2005).

The susceptibility of bull sperm chromatin to DNA fragmentation is assessed using the acridine orange fluorescent stain (AO). Because AO is a metachromatic dye and changes its fluorescence depending on incorporation into double-, or single-stranded DNA, the DFI% [ratio of the percentage of cells with denatured, single-stranded DNA to total cells acquired (both with stable, double-stranded DNA, and denatured single-stranded DNA)] can be calculated. Bernecic et al. (2021) described a methodology for the measurement of samples stained with AO. Briefly, samples were diluted to a final concentration of 15 × 10^6 sperm/ml with modified medium Tyrodes plus albumin, lactate, and pyruvate (TALP). Sperm functional assessments were performed on a CytoFlex flow cytometer from Beckman Coulter. As a positive control for DNA fragmentation, a reference sample was incubated with 0.8 M HCl for 5 min at 37 °C before assessment. Samples were prepared and stained with AO (Sigma-Aldrich, St Louis, MO, USA, final concentration 4 µg/ml) according to the protocol described by Evenson and Jost (2000). AO was excited using a 488-nm laser, and green and red fluorescence were detected with a 525/40-nm or 690/50-nm band-pass filter, respectively. Bittner-Schwerda et al. (2022) utilised the AO-based analysis of DNA stability using an Epics XL-MCL flow cytometer (Beckman Coulter). Cells were exposed to a laser beam generated by a 488 nm argon laser. Fluorescence detectors 1 (FL1) and 3 (FL3) were used for the detection of green (515–530 nm) and red fluorescence (> 630 nm), respectively. Sperm samples were diluted in TNE buffer to yield a concentration of 2 × 10^6 sperm/ml. Then, 200 µl of the suspension was treated with 400 µl of an acid-detergent solution (pH 1.2). Acridine orange (Thermo Fisher Scientific, final concentration 4 µg/ml) in a phosphate-citrate buffer was added to the sample to assess the DNA integrity, as originally described by Evenson and Jost (2001). A reference bovine sperm sample with a known DFI% was evaluated every six measurements. Validation of the staining method was done using fluorescence microscopy. Nag et al. (2021) in their study measured a DNA fragmentation index using sperm chromatin structure assay. Briefly, to a solution of 2 million spermatozoa in 100 µl of TNE buffer (0.01 mol/l of Tris-HCl, 0.15 mol/l of NaCl, and 1 mmol/l of EDTA, pH 7.4), 200 µl of acid detergent solution (0.08 N HCl, 0.1% Triton X-100; pH 1.2) were

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added and incubated for 30s at room temperature. To this, 600 µl of Acridine Orange (Sigma-Aldrich, St Louis, MO, USA) staining solution (200 mmol/l of Na_2HPO_4; 0.1 mol/l of citric acid buffer, pH 6.0; 1 mmol/l of EDTA; 150 mmol/l of NaCl; and 6 µg/ml of AO) were added and incubated for 3 min at room temperature. The samples were analysed using an analytical flow cytometer (CytoFlex S, Beckman Coulter Life Sciences, Indianapolis, IN). For each sample, a minimum of 10×10^3 events were analysed. Forward scatter (FSC), side scatter (SSC), FL1 (green fluorescence), and FL3 (red fluorescence) were measured after excitation with a blue laser (488 nm). The DFI% [ratio of the percentage of cells with denatured, single-stranded DNA to total cells acquired (both with stable, double-stranded DNA, and denatured single-stranded DNA)] was calculated for each sample using a trial version of FCS Express 7 (Denovo Software, Pasadena, USA).

Evaluation of sperm plasma membrane integrity

Many fluorochromes only stain bull spermatozoa with damaged plasma membranes. For example, propidium iodide (PI), only binds to DNA of sperm with a damaged membrane (to cells that are damaged or dead). Therefore, PI is routinely used for the analysis of sperm viability indicating plasma membrane integrity. For evaluation of sperm plasma integrity, Bittner-Schwerda et al. (2022) employed PI staining methods: immediately before analysis, semen was diluted in warm (37 °C) Tyrode's medium to produce a concentration of 5×10^6 sperm/ml. The molecular probe was added as described below, and incubation occurred for 30 min at room temperature. The PI stain was added to the diluted semen to yield final concentrations of 2.99 mM. Plasma membrane integrity was evaluated using a CytoFlex flow cytometer (Beckman Coulter Inc., Nyon, Switzerland) equipped with a 488 nm laser. The red fluorescence signal of PI-stained cells was captured using a 610/20 nm band-pass (BP) filter. Bremer et al. (2023) used this brief FC methodology: for each sample, 1 ml of fresh staining solution was prepared with 980 µl of PBS, and 0.2 µl of 2.4 mM of PI. One million sperm (volume 20 µl) were added to 1 ml of staining solution and incubated in darkness for 10 min at room temperature. Each sample was analysed in triplicate. The proportion of viable (PI^{neg}) sperm was analysed with CytoFlex Research Flow Cytometer (Beckman Coulter, Fullerton, CA, USA). The CytExpert software (v2.4, Beckman Coulter) was used to define subpopulations of interest. de Almeida et al. (2022) assessed spermatozoa plasma membrane integrity using the methodology as follows: for flow cytometry, 100 µl of sperm sample out of commercial cryopreserved 0.25 ml sperm straw containing 9.61×10^6 spermatozoa/ml was diluted in Tyrode albumin lactate pyruvate medium to a final concentration of 5×10^6 sperm/ml and kept at 37 °C. To assess sperm membrane integrity, 15 µM of Propidium Iodide probe (L0770, Sigma-Aldrich Co., St Louis, MO, USA) was added to 50 µl of semen. The sperm membrane integrity analysis was conducted in the BD Accuri C6™ flow cytometer (Becton-Dickinson, San Jose, CA, USA), equipped with an argon laser (488 nm pass) and a red laser (640 nm range). In each analysis, 10×10^3 cells were evaluated. In the study of DeJarnette et al. (2022), sperm viability was assessed using a Beckman-Coulter Cell Lab Quanta SC (Beckman Coulter, Indianapolis, IN, USA) or MACSQuant Analyzer 10 (Miltenyi Biotec, Auburn, CA, USA). After thawing a cryopreserved sample in a 0.5 ml French straw for 1 min at 37 °C in a water bath, the sample was placed in a 2 ml Eppendorf tube and incubated for 5 min with propidium iodide (PI; Sigma-Aldrich, St Louis, MO, USA). Briefly, extended semen (2 µl) was diluted in buffer (198 µl; 0.14 M TRIS, 0.14 M citric acid, 10% bovine serum albumin w/v, pH 7) containing PI (5 µg/ml) and incubated for 15 min at 35 °C in the absence of light. For flow cytometric analysis, samples were prepared in round bottom 96-well plates and a total of 5×10^3 cells/sample were analysed. Sperm were identified and gated based on size using side scatter and Coulter volume (electronic volume), and a 488 nm laser was used to excite PI, and emission spectra were collected using a 655–730 nm long pass filter. Sperm that lacked PI fluorescence were considered viable. Flow cytometric data were analysed using FlowJo procedures (FlowJo, LLC, Ashland, OR, USA). Del Prete et al. (2022) use the FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 488 nm argon-ion laser and a 635 nm red diode laser was used in this study with the following filters: 530/30 band-pass (green/FL-1), 585/42 band-pass (orange/FL-2), > 670 long

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pass (far-red/FL-3) and 661/16 band-pass (orange far red laser/FL-4). Side scatter and forward scatter in the logarithmic mode were used to back-gate and identify the sperm population. A minimum of 10×10^3 events (sperm cells) were evaluated per sample. The BD CellQuest Pro software (Becton Dickinson) was used for instrument control and data acquisition. Sperm samples diluted in 500 µl of Tyrode's medium (concentration 1×10^6 spz/ml) were stained with 3 µl of PI (final concentration: 12 mM) and incubated for 10 min at 37 °C in the dark. According to Leite et al. (2022), the flow cytometry (Guava EasyCyte Mini System, Guava® Technologies, Hayward, CA, USA) was performed as previously described by de Castro et al. (2016), with the concentration of all samples fixed at 25×10^6 sperm cells/ml after dilution in TL-semen medium. Analysis was performed, taking into account, at least 10×10^3 events per sample using FlowJo® v10.2 software (Ashland, OR, USA). The analysis of plasma membrane status was performed using a PI probe. Samples were incubated at 37 °C utilizing 0.5 mg/ml of PI for 10 min before analysis in the flow cytometer. The analysis was performed using a 488 nm excitation laser (20 mW) and emission detection at 630–650 nm for PI. Llavanera et al. (2022) described flow cytometry evaluations which were conducted using a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 488-nm argon-ion laser and the BD CellQuest Pro software v6.0 (Becton Dickinson, Milan, Italy). Emitted fluorescence from the 488 nm-laser was recorded by means of > 670 long pass (FL3) filter. Signals were logarithmically amplified and photomultiplier settings were adjusted prior to any staining protocol. Height of side scatter (SSH) and forward scatter (FSH) in logarithmic mode were used to discriminate the sperm population (gated in with a gate named 'sperm') from debris. A minimum of 10×10^3 sperm events per replicate were evaluated. PI staining was performed by incubating sperm samples with 7 µM PI (Thermo Fisher, Waltham, MA, USA) at 37 °C for 20 min in the dark. Red fluorescence from PI was recorded through FL3. Another methodology was used in the recent study of Pytlik et al. (2022). Specifically, 25 µl of frozen-thawed sperm were pipetted to 200 µl of Dulbecco's phosphate-buffered saline without divalent cations (Biosera Europe, Nuaille, France) and stained for 10 min at 38 °C in the dark with 12 µM PI (Sigma Aldrich, St Louis, MO, USA) for assessing plasma membrane damage (Savvulidi et al. 2021). Subsequently, sperm samples were analysed using a NovoCyte 3000 digital flow cytometer (Acea Biosciences, part of Agilent, Santa Clara, CA, USA). The flow cytometer was equipped with a blue (488 nm, 60 mW) laser; the PI fluorescence was collected using optical filter 675/30 (blue line, red fluorescence). The final concentration of cells during analysis was 0.8×10^6 spermatozoa/ml. The samples were run at a low speed (14 µl/min), and the fluorescence from 30×10^3 events was recorded for each sample. NovoExpress software, v1.3.0 (Acea Biosciences, part of Agilent, Santa Clara, CA, USA) was used for the automated cytometer setup and performance tracking, as well as for data acquisition. The same software was also used to analyse the acquired flow cytometry data.

In a very recent study (Salman et al. 2023), the integrity of sperm plasma membrane was assessed by the Hoechst 33258 fluorescent dye. Briefly, samples were analysed with a CyAn ADP flow cytometer (Beckman Coulter, Inc., Brea, USA) fitted with a 405 nm diode laser. The samples were prepared at 2×10^6/ml in 300 µl of PBS 0.5% bovine serum albumin with 4.5 µM of Hoechst 33258 (Thermo Fisher, Waltham, MA, USA). The samples were run through the cytometer after 15 min at 38 °C in the dark. The excitation/emission configuration for the Hoechst 33258 probe was a violet line with a 450/50 nm filter. Sample acquisition was controlled with the Summit v4.3.02 software. The acquisition was stopped after reaching 5×10^3 gated spermatozoa. Data were processed with the Weasel v3.2 software (Frank Battye, Melbourne, Australia).

For a similar purpose as PI and Hoechst 33258, 4',6-diamidino-2-phenylindole (DAPI), or fluoresceins from the broad family of SYTOX tags can be used as well. In a study of Bernecic et al. (2021), DAPI was used to estimate plasma membrane integrity. In brief, samples were diluted to 2×10^6 sperm/ml with modified TALP. Sperm functional assessments were performed on a CytoFlex flow cytometer from Beckman Coulter. The 3 µM DAPI (Sigma-Aldrich, St Louis, MO, USA; excited by 405 nm laser and detected with a 450/45 nm band-pass filter) was used for cell staining for 15 min at 37 °C before assessment.

For the SYTOX Green fluorescent probe, the Bernecic et al. (2021) described the following method. Firstly, samples were diluted to 2×10^6 sperm/ml with modified TALP. Sperm functional assessments were performed on a CytoFlex

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flow cytometer from Beckman Coulter. Samples were labelled with a final concentration of 30 nM SYTOX Green (Sigma-Aldrich, St Louis, MO, USA) for 20 min at 37 °C before assessment. SYTOX Green was excited using a 488-nm laser and detected with a 525/40-nm band-pass filter. Llavanera et al. (2022) described the usage of a SYTOX Red dead cell stain as a simple and quantitative single-step dead-cell indicator for use with red laser-equipped flow cytometers. Flow cytometry evaluations were conducted using a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 630-nm red diode laser and the BD CellQuest Pro software v6.0 (Becton Dickinson, Milan, Italy). Emitted fluorescence from the 630 nm-laser was recorded through a 661/16 band-pass filter (FL4). Signals were logarithmically amplified and photomultiplier settings were adjusted prior to any staining protocol. Height of side scatter (SSH) and forward scatter (FSH) in logarithmic mode were used to discriminate the sperm population (gated in with a gate named 'sperm') from debris. A minimum of 10×10^3 sperm events per replicate were evaluated. For SYTOX Red analysis, spermatozoa were incubated with 1 nM SYTOX Red (Thermo Fisher, Waltham, MA, USA) at 37 °C for 20 min in the dark. Red fluorescence from SYTOX Red was recorded through FL4.

Merocyanine 540 (M540) is another dye used routinely for the analysis of membrane fluidity. Namely, the M540 intercalates in the outer monolayer of the plasma membrane when the packing order of phospholipids decreases. Again, this fluorescent dye can be used for the evaluation of the quality of spermatozoa plasma membrane. Bernecic et al. (2021) described the methodology for the evaluation of plasma membrane integrity with the use of M540: samples were diluted to 2×10^6 sperm/ml with modified TALP. Sperm functional assessments were performed on a CytoFlex flow cytometer from Beckman Coulter. The 0.8 µM M540 (Sigma-Aldrich, St Louis, MO, USA; excited by 488 nm laser and detected with a 585/42 nm band-pass filter) was used for cell staining for 15 min at 37 °C before assessment.

YO-PRO-1 is a commercial name for a fluorescent dye which has been used to identify apoptotic cells. Due to plasma membrane degradation during the apoptosis (or apoptotic-like changes), apoptotic cells become permeant to YO-PRO-1 (but at the same time remain impermeant to propidium iodide and other dead cell stains). Live cells are not stained with YO-PRO-1. Therefore, YO-PRO-1 fluorescent dye also can be used for the detection of the changes in sperm plasma membrane. According to Ribas-Maynou et al. (2022b), the flow cytometry and analysis were performed using CytoFlex flow cytometer (Beckman Coulter; Fullerton, CA, USA) and CytExpert Software (Beckman Coulter; Fullerton, CA, USA). For all parameters, incubations with fluorochromes were conducted in samples adjusted at 1×10^6 sperm/ml in pre-warmed PBS, evaluating two replicates per sample and analysing 10×10^3 events per replicate. Samples were incubated with M540 (final concentration: 10 nmol/l) and YO-PRO-1 (final concentration: 31.25 nmol/l) at 38 °C for 15 min in the dark. Four subpopulations were identified: viable sperm with low membrane lipid disorder (M540-/YO-PRO-1-), non-viable sperm with low membrane lipid disorder (M540-/YO-PRO-1+), viable sperm with high membrane lipid disorder (M540+/YO-PRO-1-), and non-viable sperm with low membrane lipid disorder (M540+/YO-PRO-1+). Ribas-Maynou et al. (2022a) described the following YO-PRO-1 methodology: flow cytometry parameters were analysed using a CytoFlex flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with red, blue, and violet lasers (637, 488 and 405 nm). All incubations with fluorescent probes were conducted in samples adjusted to 1×10^6 sperm/ml in pre-warmed PBS, and two replicates were examined evaluating 10×10^3 sperm per replicate (flow rate was between 10 µl/s and 60 µl/s). Analysis of flow cytometry dot-plots was conducted through the CytExpert Software (Beckman Coulter; Fullerton, CA, USA). YO-PRO-1 Iodide was purchased from ThermoFisher (Waltham, MA, USA). YO-PRO-1 was excited with a 488-nm laser and detected with a FITC (fluorescein isothiocyanate) channel (525/40). In order to determine cell permeance to YO-PRO-1, samples were incubated in YO-PRO-1 (31.25 nmol/l) for 20 min at 38 °C before flow cytometry.

Behnam et al. (2023) reported the following methodology to determine apoptotic-like changes in bovine spermatozoa using the Annexin-V and PI kit (IQP, Groningen, Netherlands) to detect phosphatidylserine (PS) externalization. The samples were washed with calcium buffer, and then 10 µl of Annexin-V conjugated to fluorescein isothiocyanate (Annexin-V FITC 0.01 mg/ml) was added to 100 µl of sperm sample and incubated on ice

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for 20 min in a dark room. Then 10 µl of PI were added to the sperm suspension and incubated on ice for additional 10 min. Then, the suspension was evaluated using FACSCalibur with an air-cooled argon laser operated at 488 nm excitation and 15 mW (Becton Dickinson Biosciences, San Jose, CA, USA) flow cytometric set, as established by Topraggaleh et al.(2014). A minimum 10×10^3 spermatozoa were counted for each assay at a flow rate of 100 cells/s. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. For apoptosis assay, FL1 with a 530/30 nm filter was utilized to detect green fluorescence. The analysis of flow cytometry data was performed using FlowJo software (Treestar, Inc., San Carlos, CA, United States).

Evaluation of acrosomal status

Fluorescently labelled lectins, such as Peanut Agglutinin (PNA) or Pisum sativum agglutinin (PSA) can be used for the analysis of bull sperm acrosome integrity. In most cases, simple flow cytometric protocols without cell fixation are used. As such, only cells with damaged acrosomes are tagged by fluorescent lectins. Lectins themselves are not fluorescent; therefore, the lines must be labelled with any of the commercially available fluorochromes (depending on the flow cytometer that will be used for its detection). For the evaluation of acrosomal status in bull spermatozoa, several methodologies can be used. For instance, Bernecic et al. (2021) evaluated acrosomal status as follows: samples were diluted to 2×10^6 sperm/ml with modified TALP. Sperm functional assessments were performed on a CytoFlex flow cytometer from Beckman Coulter. The 0.5 µg/ml PNA-AF647 (alexa fluor 647, commercial name for fluorochrome; Sigma-Aldrich, St Louis, MO, USA; excited by 635 nm laser and detected with a 660/10 nm band-pass filter) was used for cell staining for 15 min at 37 °C before assessment. In another study (Bittner-Schwerda et al. 2022), semen was diluted in warm (37 °C) Tyrode's medium to produce a concentration of 5×10^6 sperm/ml. The molecular probe was added as described below, and incubation occurred for 30 min at room temperature. The PNA-FITC (Thermo Fisher Scientific) was added to the diluted semen to yield a final concentration of 100 µg/ml. Acrosome integrity was evaluated using a CytoFlex flow cytometer (Beckman Coulter Inc., Nyon, Switzerland) equipped with a 488 nm laser. The green fluorescence signal of PNA-FITC was captured using a 525/40 nm BP filter. In the study of Bremer et al. (2023), for each sample 1 ml of fresh staining solution was prepared with 980 µl of PBS, and 1 µl of 1 : 19 PNA linked with Alexa Fluor488 (Invitrogen, Waltham, MA, USA). One million sperm (volume 20 µl) were added to 1 ml of staining solution and incubated in darkness for 10 min at room temperature. Each sample was analysed in triplicate. The proportion of sperm with an intact or reacted acrosome was analysed with CytoFlex Research Flow Cytometer (Beckman Coulter, Fullerton, CA, USA). The CytExpert software (v2.4, Beckman Coulter) was used to define subpopulations of interest. Also, in the study of Nag et al. (2021), sperm acrosomal integrity was assessed using peanut agglutinin (PNA) tagged with fluorochrome FITC. Briefly, 1 µl of PNA-FITC (1 mM) was added to 2 million spermatozoa in 200 µl of sp TALP and incubated in the dark for 10 min at 37 ℃ before analysing in a flow cytometer. The samples were analysed using an analytical flow cytometer (CytoFlex S, Beckman Coulter Life Sciences, Indianapolis, IN). The proportion of live acrosome intact and reacted sperm populations were assessed.

Fluorescein-conjugated Pisum sativum agglutinin can also be used for the evaluation of acrosomal status. Leite et al. (2022) in their study used PSA and flow cytometry (Guava EasyCyteTM Mini System, Guava® Technologies, USA) was performed with the concentration of all samples fixed at 25×10^6 sperm cells/ml after dilution in TL-semen medium. Analysis was performed, taking into account at least 10×10^3 events per sample using FlowJo® v10.2 software. The analysis of acrosomal membrane status was made using fluorescein isothiocyanate-conjugated Pisum sativum agglutinin (FITC-PSA) probes. Samples were incubated at 37 °C utilizing 100 mg/ml of FITC-PSA for 10 min before analysis in the flow cytometer. The analysis was done using a 488 nm excitation laser (20 mW) and emission detection at 515–530 nm for FITC.

Evaluation of mitochondrial status

Such fluorescent stains from the MitoTracker family are used for the assessment of bull sperm

https://doi.org/10.17221/152/2023-CJAS

mitochondrial functionality, a very important qualitative parameter. de Almeida et al. (2022) reported the following methodology. For flow cytometry, 100 µl of sperm sample out of commercial cryopreserved 0.25 ml sperm straw containing 9.61 × 10^6 spermatozoa/ml were diluted in TALP medium to a final concentration of 5 × 10^6 sperm/ml and kept at 37 °C. To assess sperm mitochondrial functionality, 20 µM of the MitoTracker Red CMXRos probe (M-7512; Molecular Probes, Eugene, OR) were added to a sample of 50 µl semen and homogenized. The sperm membrane integrity analysis was conducted in the BD Accuri C6™ flow cytometer (Becton-Dickinson, San Jose, USA), equipped with an argon laser (488 nm pass) and a red laser (640 nm range). In each analysis, 10 × 10^3 cells were evaluated. Llavanera et al. (2022) evaluated flow cytometry results from a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 630-nm red diode laser and BD CellQuest Pro software v6.0 (Becton Dickinson). Emitted fluorescence from the 630 nm-laser was recorded through a 661/16 band-pass filter (FL4). Signals were logarithmically amplified and photomultiplier settings were adjusted prior to any staining protocol. Height of side scatter (SSH) and forward scatter (FSH) in logarithmic mode were used to discriminate the sperm population (gated in with a gate named 'sperm') from debris. A minimum of 10 × 10^3 'sperm' events per replicate were evaluated. For Mitotracker Deep Red (MTDR) analysis, samples were incubated with 0.1 µM MTDR (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 20 min in the dark. Red fluorescence from MTDR was recorded through FL4. Madeja et al. (2021) in their study described methodology using MitoTracker Green dye (probe that labels all mitochondria, not just the actives). Prior to the procedure, the sperm were fixed in 2% PFA in PBS (15 min, 37 °C), washed in phosphate-buffered saline (PBS), and centrifuged (1 min, 1 200 g). Then, the pellet was suspended in 200 µl MitoTracker Green dye solution [20 nM in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] and incubated for 60 min at 37 °C. The analyses were performed on Amnis FlowSight (Luminex, Austin, TX, USA). MitoTracker Green dye (490/516 nm excitation/emission) was excited by the 488 nm laser, and the fluorescence signal was detected in channel 2 (532/555 nm). In a study by Pytlik et al. (2022), 25 µl of frozen-thawed sperm were pipetted to 200 µl of Dulbecco's phosphate-buffered saline without divalent cations (Biosera Europe, Nuaille, France) and stained for 10 min at 38 °C in the dark with 80 nM Mitotracker Deep Red (Thermo Fisher Scientific, Waltham, MA, USA) for the assessment of active mitochondria in the live cells. Here it is important to point out that, when studying dynamic mitochondria membrane potential in the cell, the use of other dyes (such as the JC-1, see below) might be the better option. Subsequently, sperm samples were analysed using a NovoCyte 3000 digital flow cytometer (Acea Biosciences, part of Agilent, Santa Clara, CA, USA). The flow cytometer was equipped with a red (640 nm, 40 mW) laser and the Mitotracker Depp Red fluorescence was collected using optical filter 675/30 (red line, red fluorescence). The final concentration of cells during analysis was 0.8 × 10^6 spermatozoa/ml. The samples were run at a low speed (14 µl/min), and the fluorescence from 30 × 10^3 events was recorded for each sample.

Furthermore, the 5,5,6,60-tetra-chloro-1,10,3,30-tetraethylbenzimidazolyl-carbocyanine iodide probe (JC-1) selectively enters into mitochondria, forming multimers when membrane potential is high, emitting orange fluorescence. In mitochondria with low membrane potential, JC-1 maintains its monomeric form and emits green fluorescence. This very popular fluorescent dye is used in many studies for the evaluation of mitochondrial status in bull sperm cells. Several methodologies of JC-1 usage were described in recent publications. For instance, flow cytometry analysis with JC-1 was performed to evaluate mitochondrial membrane potential (Ψm) in the study of Del Prete et al. (2022). In this study, the FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) was equipped with a 488 nm argon-ion laser and a 635 nm red diode laser was used with the following filters: 530/30 band-pass (green/FL-1), 585/42 band-pass (orange/FL-2), > 670 long pass (far-red/FL-3) and 661/16 band-pass (orange far red laser/FL-4). Side scatter and forward scatter in logarithmic mode were used to back-gate and identify sperm population. A minimum of 10 × 10^3 events (sperm cells) was evaluated per sample. For JC-1 staining, an aliquot of semen was diluted with Tyrode's medium to reach a concentration of 1 × 10^6 spz/ml (500 µl) and incubated with 2.5 µl of JC-1 (in dimethyl sulfoxide; 1 mg/ml final concentration) at 37 °C for 30 min in the darkness.

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The percentage of cells with high membrane potential in the entire sperm population was calculated. In the study of Leite et al. (2022), the mitochondrial membrane potential (MMP) was evaluated using the JC-1 (Invitrogen, Waltham, MA, USA) fluorescent probe. Sperm samples were incubated in 0.5 µl of JC-1 (76.5 mM) at 37 ℃ for 5 min before analysis on the flow cytometer (Guava EasyCyte Mini System, Guava® Technologies, USA), with excitation at 488 nm (20 mW) and detection at 590 nm. Samples were classified according to the emitted fluorescence into percentages of sperm cells with relatively less (green), medium (orange), and relatively more (red) MMP. Llavanera et al. (2022) conducted flow cytometry evaluations using a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 488-nm argon-ion laser and the BD CellQuest Pro software v6.0 (Becton Dickinson). Emitted fluorescence from the 488 nm-laser was recorded by means of 530/30 band-pass (FL1) and 585/42 band-pass (FL2) filters. Signals were logarithmically amplified and photomultiplier settings were adjusted prior to any staining protocol. Height of side scatter (SSH) and forward scatter (FSH) in logarithmic mode were used to discriminate the sperm population (gated in with a gate named 'sperm') from debris. A minimum of 10 × 10^3 sperm events per replicate were evaluated. For JC-1 analysis, spermatozoa were incubated with 8.3 µM JC-1 (Thermo Fisher; Waltham, MA, USA) at 37°C for 20 min in the dark. Green fluorescence from JC-1 was recorded through FL1, and orange fluorescence from JC-1 was recorded through FL2. In the study of Madeja et al. (2021), the staining was done according to the manufacturer's protocol (T3168, Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA). The sperm samples were incubated for 60 min at 37 °C in 300 µl Sperm-TALP medium containing 8 µM of the JC-1 dye. After incubation, the samples were briefly washed in PBS to remove excess dye (1 min, 1 200 g). The analyses were performed on Amnis FlowSight (Luminex, Austin, TX, USA). In the case of JC-1, the fluorochrome was excited by the 488nm laser. Nag et al. (2021) assessed the mitochondrial membrane status of spermatozoa using JC-1 fluorochrome (Invitrogen, ThermoFischer Scientific, Waltham, MA, USA). Briefly, 20 µl of 1 mM JC-1 solution was added to 2 million spermatozoa in 200 µl, and incubated for 30 min at 37 °C in the dark, before analysing in a flow cytometer. The samples were analysed using an analytical flow cytometer (CytoFlex S, Beckman Coulter Life Sciences, Indianapolis, IN). Excitation was induced by a blue laser (488 nm). The proportion of spermatozoa with high- and low-mitochondrial membrane potential was assessed.

The 3,3'-dihexyloxacarbocyanine iodide probe [DiOC6(3)] is another fluorescent dye that can be used for the evaluation of mitochondrial status in bull sperm. At low concentrations (< 20 nM), DiOC6(3) specifically accumulates in the mitochondrial intermembrane space, and its green fluorescence increases in intensity in cells with mitochondrial membrane potential (MMP). In the recent study of Llavanera et al. (2022), the flow cytometry evaluations were conducted using a FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) equipped with a 488-nm argon-ion laser and the BD CellQuest Pro software v6.0 (Becton Dickinson). Emitted fluorescence from the 488 nm-laser was recorded by means of 530/30 band-pass (FL1). Signals were logarithmically amplified and photomultiplier settings were adjusted prior to any staining protocol. Height of side scatter (SSH) and forward scatter (FSH) in logarithmic mode were used to discriminate the sperm population from debris. A minimum of 10 × 10^3 sperm events per replicate were evaluated. DiOC6(3) staining was performed by incubating sperm samples with 20 pM DiOC6(3) (Thermo Fisher Scientific, Waltham, MA,) at 37 °C for 20 min in the dark. Green fluorescence from DiOC6(3) was recorded through FL1.

Evaluation of oxidative stress and intracellular ROS

Many fluorochromes can be used for intracellular reactive oxygen species (ROS) detection. Therefore, it is possible to evaluate the level of oxidative damage to sperm cells. For instance, the MitoSOX probe is used routinely to assess superoxide production in bull spermatozoa. To evaluate the level of oxidative stress, MitoSOX fluorescent dye was used in the study by Bernecic et al. (2021). Samples were diluted to 2 × 10^6 sperm/ml with modified TALP. Sperm functional assessments were performed on a CytoFlex flow cytometer from Beckman Coulter. Samples were labelled with a final concentration of 2.5 µM MitoSOX Red (Sigma-Aldrich, St Louis,

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MO, USA) for 20 min at 37 °C before assessment. MitoSOX Red was excited using a 488-nm laser and detected with a 585/42-nm band-pass filter.

The CellRox probe is used for reactive oxygen species detection in sperm cells. The probe penetrates the sperm cell and, when oxidized by intracellular ROS, emits an intense green fluorescence. This particular methodology was used by Leite et al. (2022): sperm samples were incubated with the probe at a concentration of 5 µM for 30 min, at 37 °C. Flow cytometry (Guava EasyCyte Mini System, Guava® Technologies, USA) was performed using procedures previously described by de Castro et al. (2016). Readings were carried out at 488 nm (20 mW) excitation and detection occurred at 515–530 nm for CellRox. Analysis was performed and included at least 10×10^3 events per sample using FlowJo® v10.2 software.

Hydroethidine (HE) is a compound that oxidizes into Ethidium (E) in the presence of O_2^-. This fluorochrome is used in order to determine the intracellular superoxide levels. The methodology was reported in the study by Ribas-Maynou et al. (2022a) where flow cytometry parameters were analysed using a CytoFlex flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with red, blue, and violet lasers (637, 488 and 405 nm). All incubations with fluorescent probes were conducted in samples adjusted to 1×10^6 sperm/ml in pre-warmed PBS, and two replicates were examined evaluating 10×10^3 sperm per replicate (flow rate was between 10 µl/s and 60 µl/s). Analysis of flow cytometry dot-plots was conducted through the CytExpert Software (Beckman Coulter; Fullerton, CA, USA). Fluorochrome Hydroethidine was purchased from ThermoFisher (Waltham, MA, USA). HE was excited with a 488-nm laser and detected through the PE channel (585/42). In order to determine intracellular superoxide levels, samples were incubated in HE (5 µmol/l) for 20 min at 38 °C.

Another methodology for evaluation of intracellular ROS using dihydroethidium (DHE) was recently reported in the study by Behnam et al. (2023). The methodology was as follows: a FACSCalibur (Becton Dickinson Biosciences, San Jose, CA, USA) flow cytometric set was used to evaluate intracellular ROS production with an air-cooled argon laser operated at 488 nm excitation and 15 mW (Topraggaleh et al. 2014). A minimum 10×10^3 spermatozoa were counted for assay at a flow rate of 100 cells/s. The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. For evaluation intracellular ROS production red fluorescence were detected with a FL2 detector (525–625 nm). The analysis of flow cytometry data was performed using FlowJO software (Treestar, Inc., San Carlos, CA, United States). The intracellular ROS was assessed using dihydroethidium (DHE; D 7008, Sigma-Aldrich, St Louis, MO, USA). DHE is oxidized by free intracellular $O_2^-\bullet$ into ethidium bromide that binds to DNA and emits red fluorescence. To detect intracellular $O_2^-\bullet$, semen samples ($1–2 \times 10^6$ sperm/ml) were incubated with 1.25 µM of DHE in the dark for 20 min at room temperature (25 °C).

The 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) is a cell-permeant compound that, in the presence of reactive oxygen species (ROS), is converted into dichlorofluorescein, emitting green fluorescence. In the recent study by Ribas-Maynou et al. (2022a), the intracellular ROS was evaluated after staining with H_2DCFDA (100 µmol/l) for 20 min at 38 °C. Flow cytometry parameters were analysed using a CytoFlex flow cytometer (Beckman Coulter, Fullerton, CA, USA), equipped with red, blue, and violet lasers (637, 488 and 405 nm). The H_2DCFDA were excited with a 488-nm laser and detected with the FITC channel (525/40). After simultaneous staining of cells with H_2DCFDA and PI (5.6 µmol/l), four subpopulations could be identified: viable spermatozoa with low levels of ROS (DCF–/PI–), viable sperm with high levels of ROS (DCF+/PI–), non-viable sperm with low levels of ROS (DCF–/PI+), and non-viable sperm with high levels of ROS (DCF+/PI+).

Evaluation of intracellular calcium

Intracellular calcium concentration in spermatozoa is assessed using Fluo 3 AM probe, which is a cell-permeant indicator that exhibits an increase of fluorescence upon Ca^{2+} binding. Intracellular calcium concentrations in spermatozoa were assessed in the study by Nag et al. (2021), using Fluo 3 AM (Invitrogen, ThermoFischer Scientific, Waltham, MA, USA). To a solution containing 2 million spermatozoa in 200 µl of sp TALP, 3 µl of Fluo 3 AM (1 mM) was added and incubated at 37 °C for 30 min. before analysing in a flow cytometer. The sperm populations were categorized

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as spermatozoa with high and low calcium levels. All the flow cytometry analyses were performed using CytoFlex S flow cytometer (Beckman Coulter Life Sciences, Indianapolis, IN). Excitation was induced by a blue laser (488 nm). Acquisitions and analyses were made using CytExpert software (Beckman Coulter). Ribas-Maynou et al. (2022a) also used Fluo 3 probe for the measurement of intracellular calcium, and samples were incubated with Fluo 3 (1.17 µmol/l) for 10 min at 38 °C before flow cytometry analysis. CytoFlex flow cytometer (Beckman Coulter, Fullerton, CA, USA) equipped with red, blue, and violet lasers (637, 488 and 405 nm) was used to excite Fluo 3 probe and collect its emission. Fluo 3 was excited with a 488-nm laser and detected with the FITC channel (525/40). After simultaneous staining of cells with Fluo 3 and PI (5.6 µmol/l), four subpopulations were identified: viable sperm with low levels of Ca^{2+} (Fluo3−/PI−), viable sperm with high levels of Ca^{2+} (Fluo3+/PI−), non-viable sperm with low levels of Ca^{2+} (Fluo3−/PI+), and non-viable sperm with high levels of Ca^{2+} (Fluo3+/PI+).

Fluorochromes for the improvement of the analysis accuracy

The presence of so-called "non-sperm events" (or debris) is one of the main problems for flow cytometric analysis of spermatozoa. Several types of particles (extender-originated particles, blood or epithelial cells, tissue, or bacteria) can be a source of debris. To improve the analysis accuracy, the debris should be discarded by gating based on differences in size and/or complexity between debris and spermatozoa. This is because the debris may show autofluorescence, or even worse, debris may be labelled by fluorochromes probes. In that case, one or more sperm populations will be overestimated (Martinez-Pastor et al. 2010). It is sometimes possible to mathematically post-process flow cytometry results to make a necessary correction. For example, Petrunkina and Harrison (2010) studied debris-caused misestimation of sperm populations, proposing a series of quite useful corrective measures. However, from the prospective of the best strategy for accurate flow cytometric analysis, sperm-specific populations should be gated via a positive fluorescent label following initial identification with side and forward scatter. The positioning of this gating to detect the sperm population is usually verified by labelling spermatozoa with Hoechst 33342, a membrane-permeable DNA-specific fluorochrome that labels every spermatozoon, serving as a counterstain (Figure 1). In this figure, the importance of using Hoechst 33342 as a counterstain is demonstrated. If the sperm-like events will be identified with only its side and forward scatter (especially, if the identification will be suboptimal, „too broad" as shown in the figure) then the population of viable spermatozoa (see the left lower quadrants in density plot 3 and 4) will be overestimated due to contamination with DNA^- debris events. On the other hand, if Hoechst 33342, as a counterstain, will be used (as shown in the histogram 2), then the DNA^- debris can be easily eliminated from the analysis. This will improve the overall analysis accuracy.

In the study by Bernecic et al. (2021), samples were diluted to 2×10^6 sperm/ml with modified TALP. Sperm functional assessments were performed on a CytoFlex flow cytometer from Beckman Coulter. Cell staining was done with 1 µg/ml Hoechst 33342 (Sigma-Aldrich, St Louis, MO, USA; excited by 405 nm laser and detected with a 450/45 nm band-pass filter). The following methodology for Hoechst 33342 staining was used in the recent study by Pytlik et al. (2022): 25 µl of frozen-thawed sperm was pipetted to 200 µl of Dulbecco's phosphate-buffered saline without divalent cations (Biosera Europe, Nuaille, France) and stained for 10 min at 38 °C in the dark with 16.2 µM Hoechst 33342 (Sigma Aldrich, St Louis, MO, USA). Subsequently, sperm samples were analysed using a NovoCyte 3000 digital flow cytometer (Acea Biosciences, part of Agilent, Santa Clara, CA, USA). The flow cytometer was equipped with violet (405 nm, 50 mW) laser; the Hoechst 33342 fluorescence was collected using optical filters 445/45 (violet laser). The final concentration of cells during analysis was 0.8×10^6 spermatozoa/ml. The samples were run at a low speed (14 µl/min), and the fluorescence from 30×10^3 events was recorded for each sample. NovoExpress software, v1.3.0 (Acea Biosciences, part of Agilent, Santa Clara, CA, USA) was used for the automated cytometer setup and performance tracking, as well as for data acquisition.

Fluorescent nucleic acid stain SYTO 60 is also used for the detection of cells and discrimination of non-cellular debris. In a very recent publication,

https://doi.org/10.17221/152/2023-CJAS

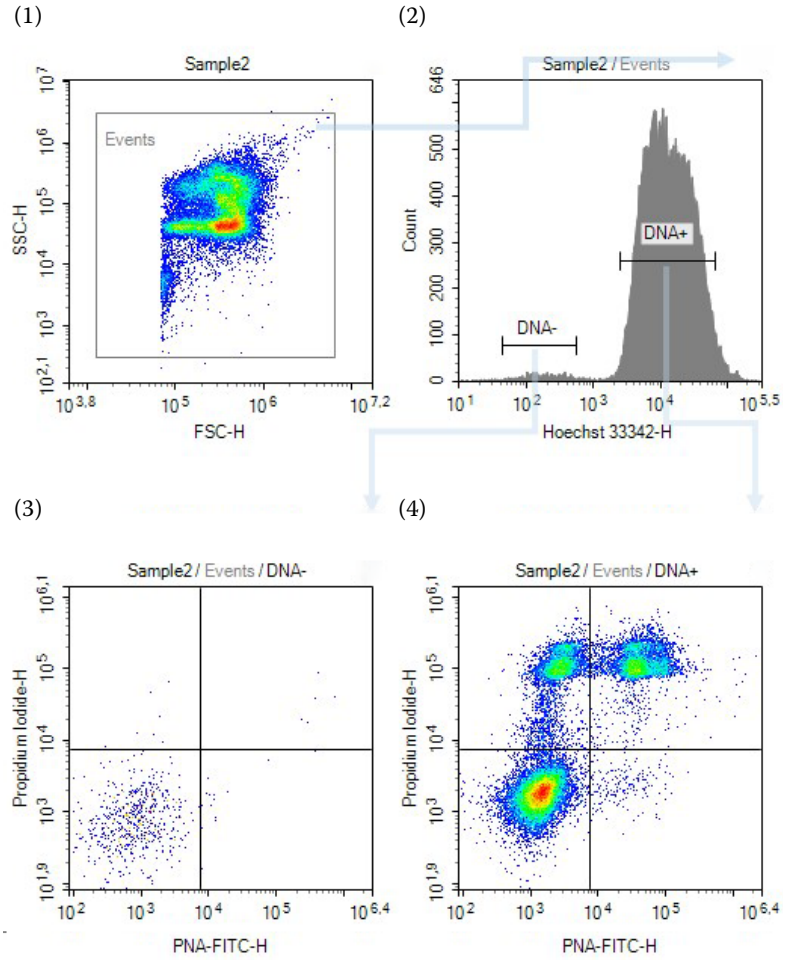

Figure 1. Improvement of the flow cytometric analysis accuracy with the use of Hoechst 33342 as a counterstain

Ram spermatozoa were gated via positive fluorescent labelling with Hoechst 33342 following intentionally suboptimal initial identification with side and forward scatter. (1) Simulation of the suboptimal cell identification based solely on the SSC and FSC parameters. (2) Demonstration of the fact that the suboptimal identified cluster of events contains actually both the DNA^- (debris) and the DNA^+ (cells) events. (3) The left lower quadrant in theory should represent only viable cells (without plasma membrane nor acrosome damage) that are, in fact, contaminated with DNA^- events. Because of this, the population of viable sperm cells might be overestimated. (4) An example of accurately identified sperm cells with the use of SSC/FSC and a positive fluorescent staining. Left lower quadrant: viable spermatozoa. Left upper quadrant: spermatozoa with damaged plasma membrane. Right upper quadrant: spermatozoa with damaged plasma membrane and damaged acrosome (double damaged sperm cells). Right lower quadrant: spermatozoa with damaged acrosome. Illustrative density plots and histograms are shown

the methodology for using SYTO 60 is described by Bremer et al. (2023). Briefly, for each sample, 1 ml of fresh staining solution was prepared with 980 µl of PBS, and 1 µl of 1:100 5 mM SYTO 60 (Invitrogen, Waltham, MA, USA). One million sperm were added to 1 ml of staining solution and incubated in darkness for 10 min at room temperature. Each sample was analysed in triplicate. The sperm cells were identified with CytoFlex Research Flow Cytometer (Beckman Coulter, Fullerton, CA, USA). The CytExpert software (v4, Beckman Coulter) was used to define subpopulations of interest.

SYBR 14 labels live sperm with green fluorescence. This fluorescent stain is usually used in combination with propidium iodide (Nagy et al. 2003). Del Prete et al. (2022) used the following strategy for SYBR 14: The FACSCalibur flow cytometer (Becton Dickinson, Milan, Italy) was equipped with a 488 nm argon-ion laser and a 635 nm red diode laser, with the following filters: 530/30 band-pass (green/FL-1), 585/42 band-pass (orange/FL-2), > 670 long pass (far-red/FL-3) and 661/16 band-pass (orange far red laser/FL-4). Side scatter and forward scatter in the logarithmic mode were used to back-gate and identify the sperm population. A minimum of 10×10^3 events (sperm cells) were evaluated per sample. The BD CellQuest Pro software (Becton Dickinson) was used for instrument control and data acquisition. Sperm samples diluted in 500 µl of Tyrode's medium (concentration 1×10^6 spz/ml) were stained with 5 µl SYBR 14 working solution (final concentration: 100 nM for cattle spermatozoa and 1 nM for buffalo) and incubated for 10 min at 37 °C in the dark. Nag et al. (2021) described in their methodology the use of SYBR 14 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) working solution, which was prepared by diluting SYBR 14 (1 mM) stock solution 50 times in dimethyl sulfoxide. SYBR 14 working solution (1.2 µl) was added to 2 million spermatozoa in 200 µl of sp TALP and incubated at 37 °C for 10 min in the dark before analysing in a flow cytometer. The samples were analysed using an analytical flow cytom-

https://doi.org/10.17221/152/2023-CJAS

eter (CytoFlex S, Beckman Coulter Life Sciences, Indianapolis, IN). Excitation was induced by a blue laser (488 nm). The proportion of membrane-intact and membrane-compromised sperm populations was assessed.

DISCUSSION

From all of the above, it is clear that there are a significant number of fluorochromes and corresponding protocols for assessing the quality of bovine semen using flow cytometry methods. In our review, we focused on only a few available, although most commonly used, fluorochromes. It should be noted that modern flow cytometers, usually equipped with several lasers, make it possible to measure combinations of several tags simultaneously (so-called multiparameter flow cytometry). However, coverage of the issue of compatibility of various tags is beyond the scope of this review. Here we aimed to highlight some methodological aspects of the use of tags to assess various qualitative parameters of sperm cells (such as DNA integrity, plasma membrane integrity, acrosomal and/or mitochondrial status, level of oxidative stress, intracellular calcium level), as well as aspects use of fluorochromes to improve the accuracy of cytometric analysis.

From the available literature, it follows that different scientific teams use different methods for describing dye concentrations. In the works presented in our review, the authors use quite different, non-comparable units of concentration. Furthermore, in most cases, authors simply did not describe the type of light source and its power in their publications accurately. This information is crucial for the appropriate comparison of methodologies and obtained flow cytometric results and must always be reported. In our review, when no information was available about the light source used, we recommend readers consider at least the type of the flow cytometer used. In general, now is the time to start an initiative for sperm cytometry, similar to MIFlowCyt: The minimum information about a flow cytometry experiment (Lee et al. 2008).

To facilitate the comparison of methodological alternatives, we provide Tables 1–7, in which concentrations are presented in the comparable micromolar concentration units (as molarity unit is recommended by the International System of Units for describing the amount of substance). For recalculation, the online molarity calculator from Tocris was used (The Tocris Molarity Calculator, available from https://www.tocris.com/resources/molarity-calculator). Molecular weight for tags was taken from the PubChem database (available from https://pubchem.ncbi.nlm.nih.gov/) or from the manufacturer. When possible, the type of flow cytometer and/or light source power is given in the tables (for light source power, the value is adopted either from the original publication directly, or from the additional communication with the authors). These tables should certainly help spermatologists when comparing alternative methodologies to select the most accurate concentration.

It is clear that the authors used the same fluorochromes, but with quite large concentration differences. It is possible that the differences are due to the type of flow cytometers used (especially, in used light sources power). On the other hand, this can be due to suboptimal excitation of used fluorochromes by the available light sources. Here we would like to stress the need for choosing fluorochromes with excitation that will fit the light sources of flow cytometer available for each particular laboratory.

Table 1. Evaluation of sperm chromatin condensation and DNA stability. Fluorochrome amounts given as a final molarity concentration. Light source power (mW)

Author	Fluorochrome	Micromolar	Cytometer type	mW
Alfadel et al. 2023	CMA3	211.3	BD FACSCANTO II	n.a.
Ribas-Maynou et al. 2022a	CMA3	10.5	CytoFlex	n.a.
Salman et al. 2023	mBBr	500	MACSQuant Analyzer 10	n.a.
Nag et al. 2021	AO	22.6	CytoFlex S	n.a.
Bittner-Schwerda et al. 2022	AO	15	Epics XL-MCL	n.a.
Bernecic et al. 2021	AO	15	CytoFlex	n.a.

n.a. = no information on light source power provided by authors

https://doi.org/10.17221/152/2023-CJAS

Table 2. Evaluation of sperm plasma membrane integrity. Fluorochrome amounts given as a final molarity concentration. Light source power (mW)

Author	Fluorochrome	Micromolar	Cytometer type	mW
Del Prete et al. 2022	PI	12 000	BD FACSCalibur	n.a.
Bittner-Schwerda et al. 2022	PI	2 990	Epics XL-MCL	n.a.
Leite et al. 2022	PI	748	Guava EasyCyte Mini System	20
Nag et al. 2021	PI	24	CytoFlex S	n.a.
de Almeida et al. 2022	PI	15	BD Accuri C6	n.a.
Pytlik et al. 2022	PI	12	NovoCyte 3000	60
DeJarnette et al. 2022	PI	7.48	Beckman-Coulter Cell Lab Quanta SC and MACSQuant Analyzer 10	n.a.
Llavanera et al. 2022	PI	7	BD FACSCalibur	n.a.
Ribas-Maynou et al. 2022a	PI	5.6	CytoFlex	n.a.
Bremer et al. 2023	PI	0.48	CytoFlex	n.a.
Salman et al. 2023	Hoechst 33258	4.5	CyAn ADP	n.a.
Bernecic et al. 2021	DAPI	3	CytoFlex	n.a.
Bernecic et al. 2021	SYTOX Green	0.03	CytoFlex	n.a.
Llavanera et al. 2022	SYTOX Red	0.001	BD FACSCalibur	n.a.
Bernecic et al. 2021	M540	0.8	CytoFlex	n.a.
Ribas-Maynou et al. 2022b	M540	0.01	CytoFlex	n.a.
Ribas-Maynou et al. 2022b	YO-PRO-1	0.031 25	CytoFlex	n.a.
Behnam et al. 2023	Annexin V	0.027 8	BD FACSCalibur	n.a.

n.a. = no information on light source power provided by authors

Table 3. Evaluation of acrosomal status. Fluorochrome amounts given as a final molarity concentration. Light source power (mW)

Author	Fluorochrome	Micromolar	Cytometer type	mW
Nag et al. 2021	PNA	5	CytoFlex S	n.a.
Bittner-Schwerda et al. 2022	PNA	0.909	Epics XL-MCL	n.a.
Bernecic et al. 2021	PNA	0.004 54	CytoFlex	n.a.
Leite et al. 2022	PSA	2 080	Guava EasyCyte Mini System	20

n.a. = no information on light source power provided by authors

Table 4. Evaluation of mitochondrial status. Fluorochrome amounts given as a final molarity concentration

Author	Fluorochrome	Micromolar	Cytometer type	mW
de Almeida et al. 2022	MitoTracker Red CMXRos	20	BD Accuri C6	n.a.
Llavanera et al. 2022	MitoTracker Deep Red	0.1	BD FACSCalibur	n.a.
Pytlik et al. 2022	MitoTracker Deep Red	0.08	NovoCyte 3000	40
Madeja et al. 2021	MitoTracker Green	0.02	Amnis FlowSight	n.a.
Leite et al. 2022	JC-1	76 500	Guava EasyCyte Mini System	20
Del Prete et al. 2022	JC-1	1 530	BD FACSCalibur	n.a.
Nag et al. 2021	JC-1	91	CytoFlex S	n.a.
Llavanera et al. 2022	JC-1	8.3	BD FACSCalibur	n.a.
Madeja et al. 2021	JC-1	8	Amnis FlowSight	n.a.
Llavanera et al. 2022	DiOC6(3)	0.000 02	BD FACSCalibur	n.a.

n.a. = no information on light source power provided by authors

https://doi.org/10.17221/152/2023-CJAS

Table 5. Evaluation of oxidative stress and intracellular ROS. Fluorochrome amounts given as a final molarity concentration. Light source power (mW)

Author	Fluorochrome	Micromolar	Cytometer type	mW
Bernecic et al. 2021	MitoSOX Red	2.5	CytoFlex	n.a.
Leite et al. 2022	CellRox	5	Guava EasyCyte Mini System	20
Ribas-Maynou et al. 2022a	HE	5	CytoFlex	n.a.
Behnam et al. 2023	DHE	1.25	BD FACSCalibur	n.a.
Ribas-Maynou et al. 2022a	H_2DCFDA	100	CytoFlex	n.a.

n.a. = no information on light source power provided by authors

Table 6. Evaluation of intracellular calcium. Fluorochrome amounts given as a final molarity concentration

Author	Fluorochrome	Micromolar	Cytometer type	mW
Nag et al. 2021	Fluo 3 AM	15	CytoFlex S	n.a.
Ribas-Maynou et al. 2022a	Fluo 3 AM	1.17	CytoFlex	n.a.

n.a. = no information on light source power provided by authors

Table 7. Fluorochromes for the improvement of the analysis accuracy. Fluorochrome amounts given as a final molarity concentration. Light source power (mW)

Author	Fluorochrome	Micromolar	Cytometer type	mW
Pytlik et al. 2022	Hoechst 33342	16.2	NovoCyte 3000	50
Bernecic et al. 2021	Hoechst 33342	2.2	CytoFlex	n.a.
Bremer et al. 2023	SYTO 60	0.05	CytoFlex	n.a.
Nag et al. 2021	SYBR 14	0.12	CytoFlex S	n.a.
Del Prete et al. 2022	SYBR 14	0.001 – 0.1*	BD FACSCalibur	n.a.

n.a. = no information on light source power provided by authors

*0.001 micromolar for buffalo, and 0.1 micromolar for cattle spermatozoa (due to observed differences in stainability between buffalo and cattle cells)

CONCLUSION

In conclusion, we would like to note that in our review, spermatologists will find, in brief, methodological approaches to the use of the most widely used fluorochromes for assessing the quality of bovine semen using flow cytometry. Our review lists 27 fluorochromes and their corresponding methodologies published over the past three years (2021–2023). From our review, spermatologists will be able to glean information about dye concentrations (in comparable, micromolar concentration units). This information will be useful in preparing their flow cytometric analysis of bovine semen quality in the most accurate way.

In addition, it should be clear from our review that different scientific groups often use the same fluorochrome, but in varying amounts that cannot be compared. On the one hand, this may reflect the fact that different flow cytometers are used (with varying degrees of light source power or light detector sensitivity). On the other hand, this may also allow us to think about some kind of future unification of the methodological approaches of the use of fluorochromes, definitely for a more optimal comparison of the obtained results. The final recommendation for researchers who prepare their study for publication: the type of light source that was used in the flow cytometric study and its power must always be clearly indicated in the study, together with a clear indication of a final molar concentration of the used fluorochromes. To eliminate the possibility of mistypes, we strongly recommend describing the SI molar unit prefix with its full name (micromolar, mil-

https://doi.org/10.17221/152/2023-CJAS

limolar, etc), and not with its abbreviation (μM, mM). This information is essential for accurate comparison of used methodologies and obtained flow cytometric results.

Acknowledgement

The authors thank students of medicine Ms. Virginia Cox and Mr. Cory Eaton, two native English speakers from the First Faculty of Medicine, Charles University in Prague for their excellent help with English language of the manuscript.

Conflict of interest

The authors declare no conflict of interests.

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Received: October 31, 2023
Accepted: December 7, 2023
Published online: December 20, 2023