Video Article

Fluorimetric Techniques for the Assessment of Sperm Membranes

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Abstract

Standard spermiograms describing sperm quality are mostly based on the physiological and visual parameters, such as ejaculate volume and concentration, motility and progressive motility, and sperm morphology and viability. However, none of these assessments is good enough to predict the semen quality. Given that maintenance of sperm viability and fertilization potential depends on membrane integrity and intracellular functionality, evaluation of these parameters might enable a better prediction of sperm fertilization competence. Here, we describe three feasible methods to evaluate sperm quality using specific fluorescent probes combined with fluorescence microscopy or flow cytometry analyses. Analyses assessed plasma membrane integrity using 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI), acrosomal membrane integrity using fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) and mitochondrial membrane integrity using 5,5',6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1). Combinations of these methods are also presented. For instance, use of annexin V combined with PI fluorochromes enables assessing apoptosis and calculating the proportion of apoptotic sperm (apoptotic index). We believe that these methodologies, which are based on examining spermatozoon membranes, are very useful for the evaluation of sperm quality.

Video Link

The video component of this article can be found at https://www.jove.com/video/58622/

Introduction

Integrity and functionality of sperm membranes are a few of the factors indicating sperm viability and fertilization potential. The plasma membrane acts as a barrier between intracellular and extracellular compartments, thereby maintaining the cellular osmotic equilibrium[1]. Any stress that induces damage to the plasma membrane integrity might impair homeostasis, reduce viability and fertilization capacity, and increase cell death. For instance, cryopreservation reduces sperm viability due to damage to its plasma membrane, as a result of temperature changes and osmotic stress[2]. We previously reported that exposing bull sperm to low concentrations of foodborne contaminants such as the pesticide atrazine, its major metabolite diaminochlorotriazine or the mycotoxin aflatoxin B1, reduces sperm viability[1,3]. This was determined by labeling the double-stranded DNA with DAPI in combination with PI, which binds to the DNA of cells with a damaged plasma membrane.

Acrosome reaction (AR) involves fusion of the outer acrosome membrane and the overlying plasma membrane resulting in the release of acrosomal enzymes[4,5]. These are essential events for zona-pellucida penetration and further merging of the sperm with the oocyte[6]. Therefore, evaluation of acrosomal membrane integrity constitutes a useful parameter to evaluate the semen quality and male fertility[7,8,9]. Several fluorescent techniques are suitable for the verification of acrosome integrity, FITC-PNA or FITC-PSA[8,10]. In our previous studies, using the patterns of FITC-PSA staining[1,3], we provided accurate definitions for (i) intact acrosome, (ii) damaged acrosome membrane and (iii) reacted acrosome. In the current report, we evaluate acrosome status using sperm-dedicated flow cytometry and compare the results to those using fluorescence microscopy.

The mitochondria are multifunctional organelles involved in, among other things, ATP synthesis, reactive oxygen species production, calcium signaling and apoptosis. Physiological dysfunctions, including male and female infertility, are associated with altered mitochondrial function[11].Sperm mitochondria are arranged in the midpiece and play a crucial role in sperm motility[12]. It is well accepted that high mitochondrial membrane potential ($\Delta\Psi m$) is associated with normal motility and high fertilization capacity[13]. In contrast, low $\Delta\Psi m$ is associated with an elevated level of reactive oxygen species and reduced fertilization rate[14]. Nonetheless, various environmental compounds, for instance endocrine disruptors, can induce cellular stress and lead to a transient increase in $\Delta\Psi m$, hyperpolarization[1,3], increased production of free radicals and eventually, apoptosis[15]. The fluorescent probe 5,5',6,6'-tetra-chloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) enables examining for example, the effects of foodborne toxins on sperm $\Delta\Psi m$[1,3].

Standard spermiograms, based on physiological and morphological parameters, are not good enough to predict semen quality. More accurate methods are required to ensure sperm quality. Here, we provide two feasible methods to determine sperm quality based on evaluations of sperm

membranes: simultaneous quadruple staining with specific fluorescent probes and fluorescence microscopy, described in our studies[1,3] and advanced sperm-dedicated flow cytometry, recently utilized in our laboratory, and already being used by others[16,17,18].

Protocol

All of the experiments were performed in accordance with the 1994 Israeli guidelines for animal welfare. Bovine sperm was supplied by commercial Israeli company for artificial insemination and breeding. Ejaculates of 11 bulls were evaluated in this study.

1. Sperm Sample Preparation

NOTE: The procedure is based on the Roth laboratory's protocol[1,3].

1. Obtain approximately 1–6 mL of bull semen in a 15 mL tube at room temperature.
2. To each 1 mL of semen, add 6 mL of prewarmed (at 37 °C) NKM buffer (110 mM NaCl, 5 mM KCl, 20 mM MOPS [3-N-morphilino propanesulfonic acid; pH 7.4]) and centrifuge for 8 min at 600 x g, 1–2 times until the supernatant is clear.
   NOTE: If sperm concentration or the initial volume are very high, split into two tubes at the first wash.
3. Immediately remove and discard the clear supernatant and leave approximately 1 cm of the supernatant above the pellet.
4. Carefully lean the tubes at a 30° angle to increase the surface area for sperm to swim up and wait 20–30 min to allow spermatozoa to swim up at 37 °C.
   NOTE: Turbidity can be seen.
5. Using a micropipette carefully, remove the upper 1 mL of the supernatant containing the motile spermatozoa to a new 1.5 mL tube.
6. Keep the sperm at 37 °C until use.
7. Estimate the sperm count using a Neubauer hemocytometer.
   NOTE: A different counting chamber can be used instead, but the counting is different.
   1. To prevent spermatozoon movement, dilute 100 µL of the motile spermatozoa with 10 mL of double distilled water (DDW) (1:100 dilution) in a 15 mL tube and mix gently.
   2. Load 10 µL of the sample into each side of the hemocytometer and coverslip. Make sure to avoid bubble formation inside the chamber as this may result in an inaccurate sperm count.
   3. Observe under a compound microscope with a 20X objective.
      NOTE: The full grid on a hemocytometer contains 9 large squares, each 1 mm^2, and the coverglass rests 0.1 mm above the floor of the chamber. Thus, the volume over the central counting area is 0.1 mm^3 or 0.1 µL. The central area of the hemocytometer contains 25 medium squares and each medium square has 16 smaller squares with single lines.
   4. Count the total number of cells found in 4 medium corner squares and the central square. For higher precision, count two chambers (both sides of the Neubauer hemocytometer) and use the average to calculate cell concentration.
   5. Calculate the sperm count by multiplying the mean number obtained by 5 (to obtain the number of cells per counting area) and by 10,000 (to obtain the number of cells per 1 mL of diluted sample). Then multiply the obtained count by the dilution factor (1:100).
      NOTE: For example, an average number of sperm counted in 5 of the 25 medium squares within the central counting area of two chambers is 150 ([152+148]/2). Thus, the mean number of sperms per chamber (or per 0.1 µL) is 150 x 5 = 750. Multiply 750 by 10,000 to obtain the number of cells per 1 mL of diluted sample (7,500,000) and then multiply by 100 (dilution factor) to obtain 75 x 10^7 cells per mL of original semen sample.

2. Technique #1: Simultaneous Assessment of Sperm Membranes Using Multiple Fluorescent Probes

NOTE: Sperm membranes (plasma, acrosomal and mitochondrial) were assessed as previously described by Celeghini et al.[10], with some modifications. Epifluorescent microscopy was used, combined with a digital camera with excitation at 450-490 nm and emission at 515-565 nm using a triple filter.

1. **Prepare stock solutions.**
   1. Prepare 0.1 mg/mL DAPI stock solution by dissolving 5 mg of DAPI in 50 mL of phosphate buffered saline (PBS). Prepare 50 µL aliquots and store at -20 °C. Before use, dilute the stock solution with PBS at 1:10 (working solution; 10 µg/mL).
   2. Prepare 1 mg/mL FITC–PSA stock solution by dissolving 1 mg of FITC–PSA in 1 mL of PBS. Prepare 50 µL aliquots and store at -20 °C. Before use, dilute the stock solution with PBS at 1:10 (working solution; 100 µg/mL).
   3. Prepare 1 mg/mL JC-1 stock solution by dissolving 1 mg of JC-1 in 1 mL of dimethyl sulfoxide (DMSO). Prepare 10 µL aliquots and store at -20 °C. Before use, dilute the stock solution with DMSO at 1:10 (working solution; 0.1 mg/mL).
   4. Prepare the PI stock solution by dissolving 10 mg of PI in 400 µL of PBS (giving 2.5 mg/mL). Store at +4 °C. Dilute stock 1 with PBS at 1:20 (working solution; 0.125 mg/mL). Store at +4 °C as a stock solution.
      Caution: PI is a potential mutagen and should be handled with care. The dye must be disposed of safely and in accordance with applicable local regulations.

2. Transfer 133 µL of the motile spermatozoa (step 1.5) to a new 1.5 mL tube (25 x 10^6 sperm/mL).
   NOTE: If the sample concentration is higher, dilute it in NKM buffer to achieve the required concentration; if the sample concentration of the swim up sample is lower, centrifuge the obtained supernatant after swimming up at 1,000 x g for 5 min, remove 0.5 mL of the supernatant and count the sperm again.
3. Add 17 µL of DAPI (working solution) and incubate for 10 min at 37 °C.
4. Centrifuge at 1,000 x g for 5 min and discard the supernatant.
5. To the pellet, add 100 µL of NKM buffer.

6. Add 50 µL of FITC–PSA, 2 µL of JC-1 and 3 µL of PI (working solutions) and incubate for 10 min at 37 °C.
7. Centrifuge at 1,000 x g for 5 min and remove the supernatant.
8. To the pellet, add 40 µL of NKM buffer and resuspend by pipetting.
9. Transfer 10 µL of the sample to a glass slide, smear and coverslip.
10. **Visualize immediately by epifluorescence microscopy (use 40x objective) with a triple filter, equipped with a digital camera and capture an image separately for each filter.**
    NOTE: There is no significance to the order of filters visualized.
    1. Visualize under DAPI channel with excitation at 358 nm and emission at 461 nm.
    2. Visualize under FITC channel for green monomers with excitation at 450–490 nm and emission at 515–565 nm.
    3. Visualize under PI channel for red aggregates with excitation at 488 nm and emission at 590 nm.
    4. Visualize under JC-1 red aggregates with excitation at 559 nm, and emission in the range of 574–627 nm; JC-1 green monomers with excitation at 488 nm and emission in the range of 500–535 nm.

11. Merge the three images received from the filters in JPG/JPEG format, using the "merge" option of the camera software.
12. Open the merged image with "Paint" tool and use the brush option to mark counted spermatozoa.
13. **Classify spermatozoa based on the fluorescence emitted from each probe:**
    1. In general evaluate at least 200 spermatozoa per slide—all cells appear blue (DAPI).
    2. Evaluate the viability by counting dead cells, which appear purple (PI [red] + DAPI [blue]) and calculate the percentage of dead cells (dead cells/total counted cells x 100).
    3. Evaluate acrosome status using the patterns of fluorescent staining (FITC–PSA). Calculate the percentages of the different patterns (intact, damaged or reacted acrosome cells/total counted cells x 100).
       NOTE: Damaged acrosomal membrane appears as a fully stained, green acrosome cap; reacted acrosomal membrane shows residual green equatorial or upper staining; cells containing intact acrosomal membrane will not exhibit any green staining of the acrosomal region.
    4. Evaluate ΔΨm by distinguishing spermatozoa with high ΔΨm, which exhibit a red-stained midpiece, and spermatozoa with low ΔΨm which exhibit a green-stained midpiece. Count red and green midpieces separately and calculate their ratio (red/green).

3. Technique #2: Assessment of Sperm Membranes with Ready-To-Use Kits and Flow Cytometry

NOTE: Assessment of plasma membrane integrity, mitochondrial membrane potential and acrosomal membrane integrity was performed with ready-to-use flow cytometry kits containing lyophilized fluorochromes in each well. The procedure was performed according to the manufacturers with some modifications.

1. **Plasma membrane integrity evaluation**
    1. Take the desired number of wells from the package of viability and concentration kit (PI and SYbr14), transfer them to the working base and cover with a flexible lid (protect from light).
    2. Add 199 µL of buffered solution for cytometry per well.
    3. Add 1 µL of homogeneous semen at 57 x 10^6/mL (57,000 cells per well) and homogenize by pipetting.
    4. Cover the plate with the black lid.
    5. Incubate for 10 min at 37 °C protected from light.
    6. Run the sample through the flow cytometer with the setting 'viability'.

2. **Mitochondrial membrane potential**
    1. Take the desired number of wells from the package of mitochondrial activity kit (JC-1), transfer them to the working base and cover with a flexible lid (protect from light).
    2. Add 10 µL of absolute ethanol per well and pipette to resuspend the powder present within the well.
    3. Add 190 µL of PBS per well and homogenize by pipetting.
    4. Add 0.75 µL of homogeneous semen at 57 x 10^6/mL (50,000 cells per well) and homogenize by pipetting.
    5. Cover the plate with the black lid.
    6. Incubate for 30 min at 37 °C protected from light.
    7. Run the sample through the flow cytometer with the setting 'mitochondrial activity'.

3. **Acrosomal membrane integrity**
    NOTE: FITC–PSA staining (see Technique #1) enables the evaluation of 3 acrosome categories (intact acrosome, reacted acrosome and damaged acrosome). Using the flow cytometer and viability & acrosome integrity kit (PI and FITC–PNA), the spermatozoa are separated into these 3 categories.
    1. Take the desired number of wells from the package of viability & acrosome integrity kit, transfer them to the working base and cover with a flexible lid (protect from light).
    2. Add 200 µL of buffered solution for cytometry per well.
    3. Add 0.7 µL of homogeneous semen at 57 x 10^6/mL (40,000 cells per well) and homogenize by pipetting.
    4. Cover the plate with the black lid.
    5. Incubate for 45 min at 37 °C protected from light.
    6. Run the sample through the flow cytometer with the setting 'InCyte'.
    7. Analyze the resultant histogram by gating three marker areas according to fluorescence intensity, representing negligible, low-fluorescing cells with intact, unstained acrosome (R1), low-fluorescing cells with residual stained part of the acrosome (R2) and highly fluorescing cells with disrupted acrosome (R3).

NOTE: Use the "analyzing files acquired using other modules" section in the instrument user guide in order to create the three regions (R1, R2, R3).

4. Technique #3: Assessment of Sperm Membranes Using Fluorescent Probes and Flow Cytometry

NOTE: Use of annexin V combined with PI fluorochromes enables assessing apoptosis and calculating the proportion of apoptotic sperm (apoptotic index).

1. Prepare 1x annexin V binding buffer from 20x stock solution (dilute 500 µL of annexin V binding buffer 20x stock solution with 9.5 mL of sterile distilled water).
2. Estimate the sperm count using a Neubauer hemocytometer as described in section 1.7.
3. Wash 10^6 spermatozoa in 1 mL of 1x annexin V binding buffer and centrifuge at 300 x *g* for 10 min.
4. Aspirate the supernatant completely.
5. Resuspend the pellet in 100 µL of 1x annexin V binding buffer.
6. Add 10 µL of annexin V conjugated to FITC.
7. Mix well and incubate for 15 min in the dark at room temperature.
8. Wash spermatozoa by adding 1 mL of 1x annexin V binding buffer per 10^6 cells and centrifuge at 300 x *g* for 10 min.
9. Aspirate the supernatant completely.
10. Resuspend the cell pellet in 500 µL of 1x annexin V binding buffer per 10^6 total cells.
11. Add 1 µg/mL PI immediately prior to analysis with a flow cytometer.
12. Run the sample through the flow cytometer set on 'InCyte'.

Representative Results

Figure 1 shows simultaneous fluorimetric assessment of sperm membranes (plasma, acrosomal and mitochondrial) using PI, DAPI, FITC-PSA and JC-1. Assessment of sperm membranes using simultaneous staining with four fluorescent probes allows, for example, evaluating the proportion of sperm in each category—live *vs.* dead; high *vs.* low ΔΨm; intact *vs.* damaged acrosome—simultaneously for each spermatozoon.

Figure 2 presents results of sperm membrane evaluation using fluorimetric probes. Only semen that contained at least 80% motile spermatozoa were used in the experiment. At least 200 cells were examined per bull. It was possible to evaluate the differences in sperm sample quality in terms of membrane integrity. For example, the ejaculate of bull no. 7 had a relatively low percentage of dead cells, a low proportion of sperm with pseudo reacted acrosome and higher mitochondrial membrane potential, as compared to the ejaculate of bull no. 1.

Figure 3 shows representative samples evaluated for viability (**Figure 3A-3C**) and mitochondrial activity (**Figure 3D-3F**). Fluorescence intensities of the samples were evaluated by a dedicated microcapillary sperm flow cytometer, with dedicated software. This flow cytometer contains one solid-phase blue laser (448 nm) and two photodiodes: forward scatter and side scatter. It specifically measures sperm emission properties with three photomultiplier tubes (green: 525/30 nm, yellow: 583/26 nm; red: 655/50 nm) and accommodates optical filters and splitters[16]. It enables evaluation of 5,000 spermatozoa per analysis.

The viability evaluation kit contains a probe with differential permeability to viable (intact plasma membrane) and dead (damaged plasma membrane) spermatozoa (**Figure 3C**). Sperm ΔΨm was assessed using a kit that distinguishes between polarized mitochondrial membrane (fluorescence appearing in orange) and depolarized mitochondrial membrane (fluorescence appearing in green) (**Figure 3F**).

Figure 4 presents an evaluation of acrosome integrity performed with the ready-to-use kit, read with the flow cytometry (**Figures 4A-4C**), dividing the resultant histogram of gated spermatozoa into three marker areas, representing negligible low-fluorescing cells with intact, unstained acrosome (R1), low-fluorescing cells with residual stained part of the acrosome (R2), and highly fluorescing cells with disrupted acrosome (R3).

Table 1 presents a comparison of the two fluorimetric techniques for assessment of sperm membranes. The same sperm samples from three different bulls were evaluated for viability, mitochondrial membrane potential (ΔΨm) and acrosome integrity using simultaneous quadruple staining as well as flow cytometry. This comparison is highly important, as it shows the matching results using each of the two techniques. Data were analyzed by an analysis and Student's t-test. No statistically significant differences were observed.

Figure 5 shows a representative sample evaluated for apoptosis using annexin V (AV) and propidium iodide (PI) fluorochromes. Use of these two probes enables distinguishing among four patterns indicating viable cells (AV-, PI-), early apoptotic cells (AV+, PI-), apoptotic cells (AV+, PI+) and necrotic cells (AV-, PI+).
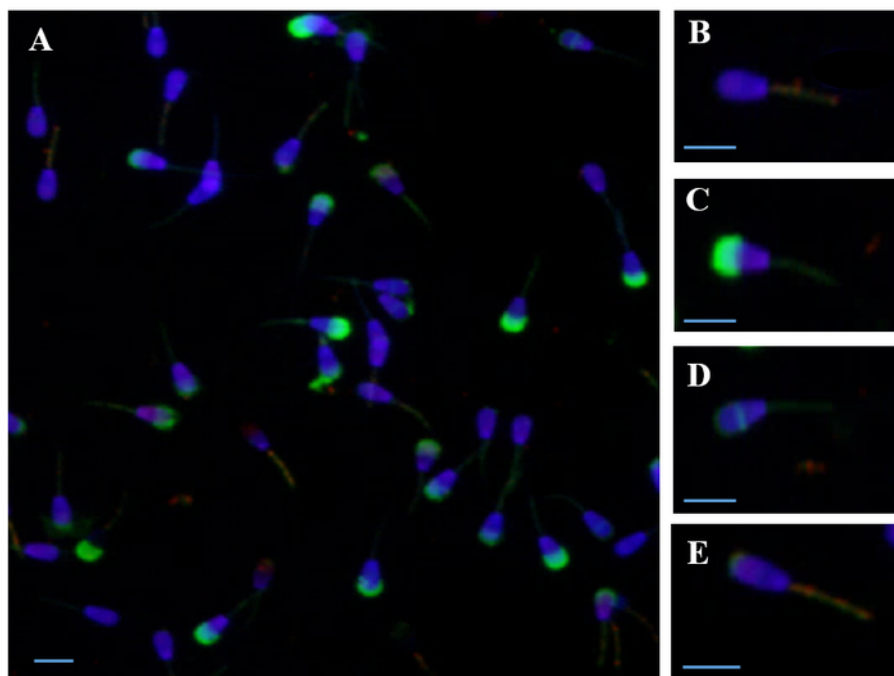
Figure 1: Epifluorescence photomicrography of spermatozoa stained simultaneously with several fluorescent probes. (**A**) Simultaneous staining with four probes PI, DAPI, FITC-PSA and JC-1) (**B**) Live spermatozoon with DAPI staining of nucleus and high mitochondrial membrane potential (ΔΨm), stained with JC-1 probe. (**C**) Dead spermatozoon with damaged plasma membrane stained with PI probe, damaged acrosome stained with FITC-PSA probe and low ΔΨm. (**D**) Live, acrosome-reacted spermatozoon with residual equatorial staining and low ΔΨm. (**E**) Live, acrosome-reacted spermatozoon with residual upper staining and high ΔΨm. Scale bars = 10 µm. Please click here to view a larger version of this figure.
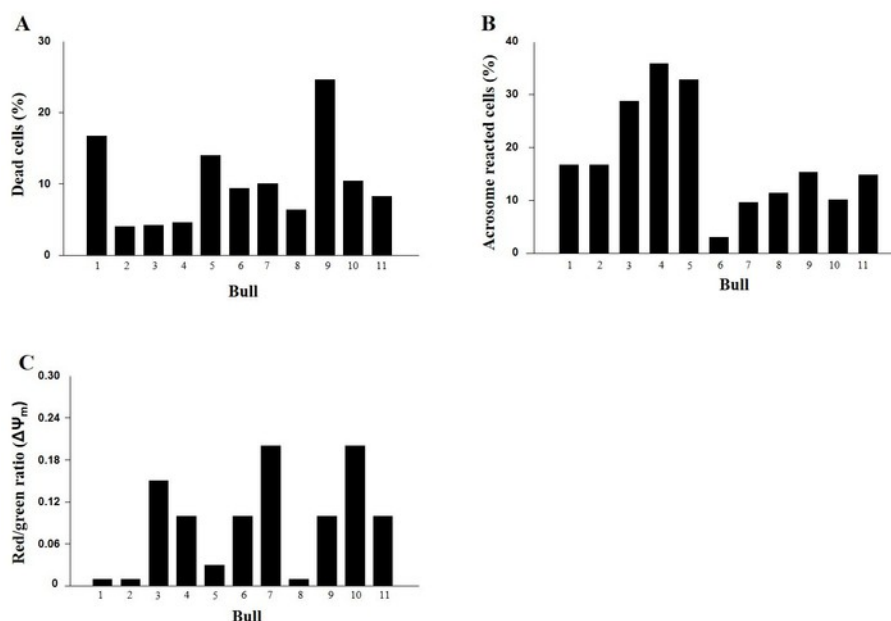

Figure 2: Evaluation of bull sperm membranes using fluorimetric probes. (**A**) Sperm viability was determined with fluorescent probes 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI). (**B**) Acrosome status was determined according to FITC-PSA staining patterns. Presented are the proportion of spermatozoa with reacted acrosome. (**C**) Mitochondrial membrane potential (ΔΨm) was evaluated using with JC-1 fluorescent probe and presented as the ratio between mean proportion of red-stained (high potential) and green-stained (low potential) sperm. Data are presented as percent of cells out of total evaluated cells. At least 200 spermatozoa were analyzed per bull. Please click here to view a larger version of this figure.
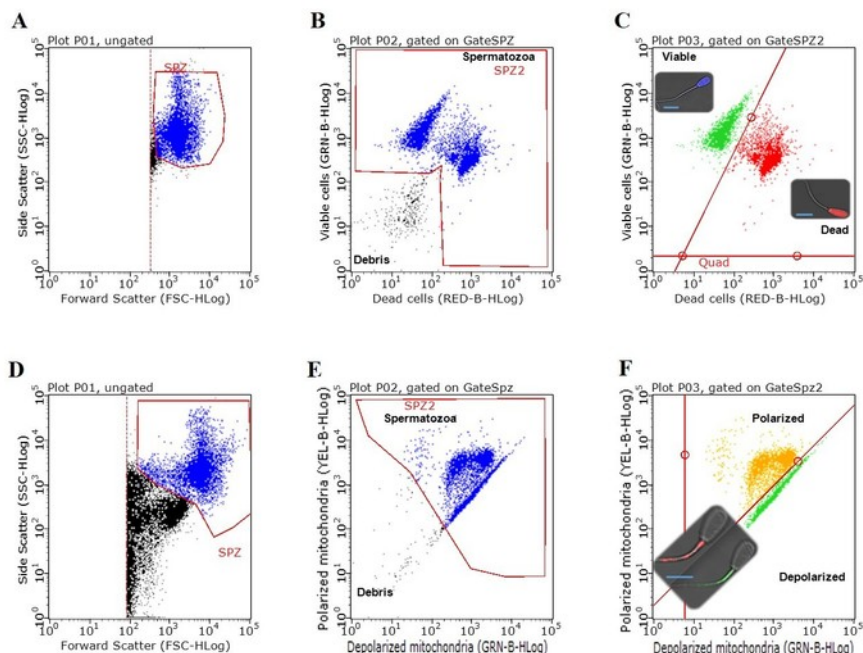
Figure 3: Viability (A-C) and mitochondrial activity (D-F) fluorescence assessment of representative samples measured by EasyCyte flow cytometer. Histograms represent ungated spermatozoa and debris (**A, D**), gated spermatozoa (**B, E**), distribution of spermatozoa to viable (green) and dead (red) cells (**C**), and distribution of spermatozoa to polarized (yellow) and depolarized (green) mitochondrial membrane (**F**). Scale bars = 10 μm. Please click here to view a larger version of this figure.
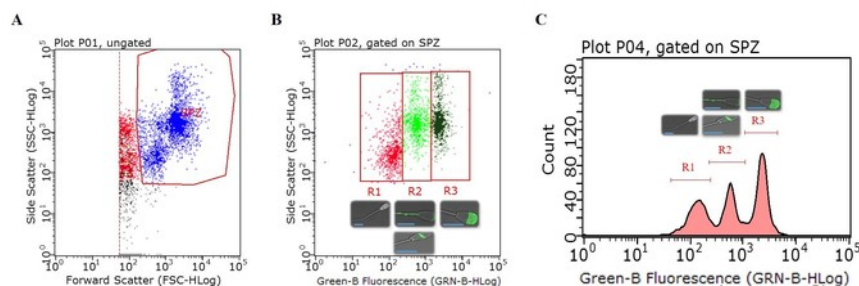

Figure 4: Fluorescence assessment of acrosome integrity of representative samples measured by EasyCyte flow cytometer. (**A**) Histogram of ungated spermatozoa and debris. (**B**, **C**) Histograms of gated spermatozoa with evaluation of acrosome integrity performed with ready-to-use kit, read with adapted setting 'InCyte', dividing the resultant histogram of gated spermatozoa into three marker areas, representing negligible, low-fluorescing cells with intact, unstained acrosome (R1), low-fluorescing cells with residual stained part of the acrosome (R2) and highly fluorescing cells with disrupted acrosome (R3). Scale bars = 10 μm. Please click here to view a larger version of this figure.

	Average No of Cells	Vialbility		Mitochondrial Membrane potential			Acrosome Integrity		
		Viable	Dead	Depolarized	Polarized	Red/Green Ratio	Intact Acrosome	Reacted Acrosome	Disrupted Acrosome
Quadruple Staining	253	32.7 ± 1.53%	67.3 ± 1.53%	65.7 ± 2.25%	34.3 ± 2.52%	0.5 ± 0.06	37.3 ± 7.2%	38.0 ± 5.7%	24.3 ± 3.0%
Flow Cytomtery	5,000	32.3 ± 2.08%	67.7 ± 2.08%	65.0 ± 1.00%	35.0 ± 1.00%	0.5 ± 0.02	39.5 ± 5.7%	39.5 ± 6.5%	21.0 ± 8.0%

Table 1: Comparison of the two fluorimetric techniques for assessment of sperm membranes. The same sperm samples were evaluated for viability, mitochondrial membrane potential and acrosome integrity using simultaneous quadruple staining and flow cytometry. Data are presented as mean proportion ± SD of the examined cells, calculated for 3 replicates.
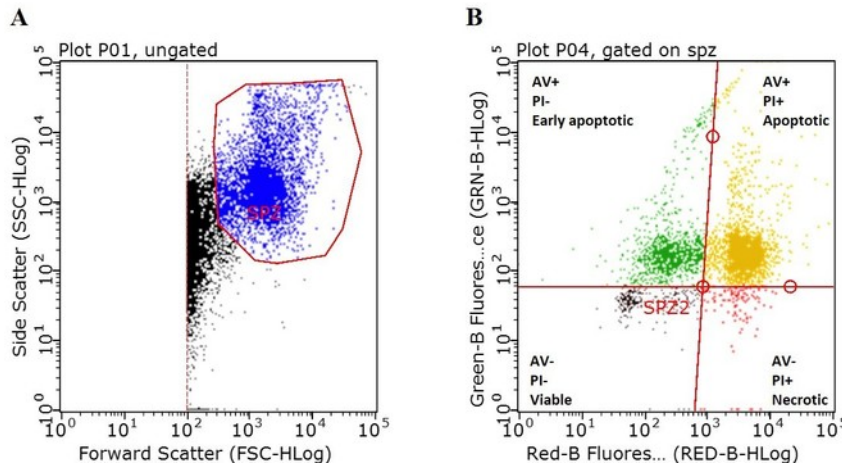
Figure 5: Annexin V and PI fluorescence of a representative sample measured by a flow cytometer. Histograms represent (**A**) ungated spermatozoa and debris and (**B**) distribution of the gated spermatozoa to early apoptotic (AV+, PI-), apoptotic (AV+, PI+), viable (AV-, PI-) and necrotic (AV-, PI+) cells. Please click here to view a larger version of this figure.

Discussion

Sperm fertilization potential depends on multiple factors reflecting its quality. A high concentration of spermatozoa and a high proportion of highly progressively motile spermatozoa might be considered high-quality semen. Nevertheless, such an evaluation does not take into account other cellular and functional parameters. The use of 'bench-top' microcapillary flow cytometer can be easily adapted to evaluation of various sperm structures using fluorescent probes, as previously shown by others[17] and demonstrated herein (Technique #3). For example, sperm acrosome integrity is highly important for the occurrence of successful natural fertilization and therefore, precise evaluation of acrosomal status is warranted. Such an evaluation can be easily performed by classification of acrosome status using the patterns of fluorescent staining (FITC-PSA, FITC-PNA, i.e., Technique #1, as previously described)[1,3]. In particular, it is highly important to determine the proportion of sperm with intact acrosome (i.e., exhibits an unstained acrosome) relative to those with damaged acrosome. With respect to the latter, sperm with damaged acrosome can exhibit (i) a fully stained acrosomal cap, which indicates that the membrane is damaged, enabling the dye to flow through the membrane into the acrosome vesicle; (ii) acrosome-reacted sperm that exhibit only residual acrosome content, indicating that the AR has already occurred (i.e., pseudo AR). It should be noted that such an evaluation can also be performed with the dedicated flow cytometer.

The ready-to-use viability & acrosome integrity kit defines both sperm viability (viable or dead) and acrosomal integrity (intact or disrupted). Here, we suggest using the dedicated flow cytometer to define the three aforementioned acrosomal statuses (i.e., intact, damaged, reacted). We adapted the microcapillary flow cytometer platform for more accurate evaluation, which identifies the acrosome-reacted sperm (i.e., low fluorescence) while excluding them from those with disrupted acrosome (high fluorescence), rather than including them with those having an intact acrosome. This gives an accurate proportion of sperm with functional or nonfunctional acrosome. Sperm with reacted acrosome as well as disrupted acrosomal membrane have lost their ability to fertilize the oocyte. Furthermore, accurate analysis might shed light on the mechanism underlying acrosome alteration, i.e., damaged acrosome membrane vs. pseudo acrosome activation.

We compared the results obtained with Technique #1 and Technique #2, and found great compatibility between them, in particular in the evaluation of viability and ΔΨm (**Table 1**). One of the main advantages of using the the dedicated flow cytometer is the large number of evaluated spermatozoa relative to the small number of spermatozoa that are evaluated in practice by fluorescence microscopy and probes (thousands vs. hundreds, respectively). Moreover, the latter procedure is time-consuming and subjective, even when performed by an experienced observer. As flow cytometry only detects particle-associated fluorescence, there is no need to wash the unbound probe from the solution, which is a time-consuming step[17]. On the other hand, the fluorimetric assessment of sperm membranes described in Technique #1 enables simultaneous assessment of multiple membranes. We were able to use as many as four fluorescent probes together[1,3].

Finally, it should be noted that the dedicated flow cytometer was developed as an open assay module, providing all of the basic tools for sample acquisition and data analysis. The acquisition function enables collecting various types of information from a cell sample and therefore allows adaptation for more accurate evaluation, as shown here for acrosome status and apoptotic index.

In conclusion, the methodologies described in this paper are very useful for the evaluation of semen quality. Examining spermatozoon membranes is highly important for determining sperm fertilization competence.

Disclosures

The authors declare that there are no conflicts of interest.

Acknowledgements

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