Probing the Structure and Function of Mammalian Sperm using Optical and Fluorescence Microscopy

João Ramalho-Santos*, Alexandra Amaral, Ana Paula Sousa, Ana Sofia Rodrigues, Luís Martins, Marta Baptista, Paula C. Mota, Renata Tavares, Sandra Amaral and **Sandra Gamboa**

Center for Neuroscience and Cell Biology, Department of Zoology, University of Coimbra
3004-517 Coimbra, Portugal.

Different aspects of sperm function can be monitored with a wide array of microscopy techniques, from simple optical microscopy to sophisticated fluorescence microscopy (epifluorescence and confocal), and the level of analysis may be adjusted and complemented according to specific needs. Cursory assessments involving sperm number and motility can be easily performed, while more detailed analyses regarding the status of sperm DNA and mitochondria, or the presence of possible apoptotic markers requires more elaborate protocols. Herein, we detail several methods routinely used to probe sperm structure and function, using a variety of microscopy techniques.

Keywords: Sperm; Fertilization; Reproduction; Immunocytochemistry

1. Introduction

The mammalian sperm is a deceptively simple, and terminally differentiated, cell [1]. Although it seems to have a limited array of functional features, in essence to deliver an intact haploid genome to an oocyte at fertilization, these functions touch many important points in physiology and cellular and molecular biology, with implications for animal production, (in)fertility and toxicology, to name but a few aspects.

A functional sperm is composed of three main regions: the head, the midpiece, and the tail. The head contains the nucleus, in which sperm-specific DNA binding proteins, called protamines, have replaced histones. The presence of protamines allows for tighter chromatin packaging, and, together with the loss of most of the typical cell organelles and cytoplasm that takes place during spermiogenesis, this is thought to have a role in reducing cell volume and increasing the sperm's aerodynamic properties, thus potentially facilitating fertilization. It should be noted that these characteristics are also widely believed to render the sperm transcriptionally inactive, or at least translationally impaired. Overlaying the nucleus is a large secretory vesicle, the **acrosome**. This vesicle contains hydrolitic enzymes thought to aid in sperm penetration through oocyte-protecting layers, namely the translucent glycoprotein-based *zona pellucida*. Release of acrosomal contents, an exocytotic process dubbed the **acrosome reaction**, must only take place in the vicinity of an oocyte, and the lack of an acrosome in any other circumstance signals that the sperm will likely not be fully functional. However, before the sperm can undergo the acrosome reaction it must be primed by a series of poorly understood maturation steps involving tightly regulated intracellular signaling and protein phosphorylation (characteristically on tyrosine residues), which comprise what is called sperm **capacitation**. The sperm tail (or flagellum) is paramount for sperm motility, and is comprised of an axoneme containing a typical 9+2 microtubular arrangement, consisting of nine doublet pairs of peripheral microtubules, arranged in circle around a central pair. Other protein structures (the outer dense fibers and fibrous sheath) are arranged around the axoneme, adding strength and resistance to the tail. The sperm midpiece, as its name indicates, apparently connects the head and the tail, although it in fact consists of a variable number of mitochondria wrapped helically around the anterior portion of the flagellum. It has been assumed that the role of these mitochondria might be to

* Corresponding author: João Ramalho-Santos, Center for Neuroscience and Cell Biology, Department of Zoology, University of Coimbra, 3004-517 COIMBRA, PORTUGAL. TEL: + 351(239)-855 760; FAX: + 351(239)-855 789; email: jramalho@ci.uc.pt

provide ATP for sperm movement through oxidative phosphorylation. However, it is also possible that the necessary ATP is produced mostly by glycolitic pathways throughout the flagellum, with mitochondrial ATP required only in specific circumstances. It is also very important to note that there are many species-specific differences, and what holds true for one species may not translate well (or at all) to another. A human sperm is only 60 μm long, while a rat sperm can reach 190 μm. Rodent sperm are also distinctive for their hook-shaped heads. It is quite likely that these structural distinctions have physiological (species-specific) implications.

Despite the extensive knowledge on spermatology, a definition of what constitutes a "good" spermatozoon (i.e. a male gamete with high fertilization potential) remains elusive. Besides the already mentioned species-specific differences, the fact that ejaculates from the same male may vary according to several factors, and that they are always very heterogeneous, containing both functional sperm and defective cells, is the main obstacle for proper analysis. However, there are several aspects of sperm biology that can be monitored, and which can give clear indications on the potential fertility of a given sample. Furthermore, the analysis of sperm structure and function may be adjusted and complemented according to specific needs, be they clinical, teaching or research; and several methods can be chosen according to both the purpose of the assay, and the available resources.

In this Chapter we describe some of the methods used to assess sperm function from several mammalian species at several levels, from sperm motility and vitality, to sperm DNA, mitochondrial and acrosomal status. Besides direct clinical and research applications, these methods have been successfully used to convey information to both professionals and students (undergraduate and graduate) of several types of backgrounds and with diverse interests, from Researchers, Veterinarians, and Physicians, to Engineers and technicians.

2. Classical sperm parameters and sperm vitality

Classical analysis of a sperm sample focuses on three parameters: sperm count, sperm motility and sperm morphology. Indeed, the World Health Organization (WHO) has established threshold values for these parameters, below which the sample is deemed abnormal, and the likelihood of human male infertility is considered high. The values defining a normal human sample are at least 20 million sperm/ml, at least 50% of motile cells, and at least 14% of morphologically normal sperm [2], and they can be easily determined in any species using simple optical microscopy or colorimetric techniques.

2.1. Sperm concentration, motility and morphology

Besides its clinical importance in humans, determining sperm concentration is essential to prepare effective doses for Artificial Insemination (AI) or to determine the effect of xenobiotics on spermatogenesis. One of the most accepted methods to assess sperm concentration of a semen sample is the optical microscopy-based hemocytometer method using an appropriate counting chamber, a classical method used also for other cell types. To facilitate counting the cells are immobilized using either a fixative and/or osmotic shock. The suspension must be meticulously, but gently, mixed, and each side of the counting chamber carefully loaded without being under- or overfilled. Counts should be performed in both sides of the chamber using a 400x magnification. In the improved Neubauer® chamber the counting area in each side of the chamber is a square with 1 mm^2 and a depth of 0.1 mm, giving a total volume of $0.1mm^3$ for each side. This square is divided in smaller squares with a volume of 0.004 mm^3. Loose tails and other cell types are not counted, and sperm on a line are only counted if they are on the lower or left-hand sides of the square being counted. Sperm concentration (expressed as 10^6 sperm/ml) is calculated dividing the total number of sperm counted by the volume and multiplying by the dilution factor used to prepare the sample. The hemocytometer method can also calibrate spectrophotometers, which are then used to quickly calculate bull, boar and stallion sperm concentrations for AI. In this case concentration is given by the turbidity due to the cells in suspension, a much quicker assessment than what is provided by the hemocytometer method [3].

Sperm motility should be monitored using a phase contrast optical microscope and a heated environment, whenever possible. The sample is evaluated according to the percentage of cells that display movement. However, there are various types of sperm movement (vibration, circulatory movement, oscillatory movement, slow and fast progressive motion), and different classifications may be employed for different species.

There are several methods to monitor sperm morphology. A simple method to monitor the main regions of the spermatozoon (i.e. head, neck/midpiece, tail/principal piece) consists of a smear of semen with India ink [3]. The black background contrasts with the uncolored sperm, and morphology is easy to determine using ordinary light microscopy (Figure 1C). The slides must be warmed in a heated stage and at least 10 µl of the semen sample should be put side by side with 10 µl of India ink. The smear is prepared mixing the 2 drops for 10 s, incubating for an additional 50 s, spreading the sample with a coverslip, and allowing the slide to dry. Minimal force must be applied while spreading; otherwise the sperm tails may be broken. Other methods to assess sperm morphology rely on optical stains using different types of dyes. Diff-Quik (Dade Behring Inc., Newark, NJ) is a simple and fast optical staining technique, composed by a fixative (methanol), an anionic/acidic dye (eosin), which stains positively charged/basic proteins red, and by a thiazin-like stain (methylene blue and/or its derivatives), which stains DNA blue [4]. Sperm smears are performed using 10 µl of a sperm suspension and allowed to air dry. Slides are immersed, sequentially in each one of the solutions, and rapidly dipped in water. They are then allowed to air dry, mounted in a rapid mounting media, and observed in a bright field microscope (Figure 1A,B). Whatever the chosen method, a total of 200 sperm cells should be observed with a 1000x magnification (100x objective under oil immersion). The number of normal and morphological defective sperm should be registered, as well as the type of defect, and the cell region where it is detected (i.e., misshapen heads, curled tails, doubled headed sperm, etc). It should be noted that there is a hierarchical classification of sperm defects for each species, which should be consulted when performing an analysis (i.e. some defects are deemed more limitative to sperm function than others).

2.2. Sperm Viability

Although classical semen parameters remain a standard in sperm analysis, often the results have very little predictive power, and do not fully indicate the quality of a given sample. Sperm viability may give important additional information, by discriminating live from dead sperm. Two techniques can be used: the optical eosin-nigrosin assay and the fluorescence live-dead assay, which employ, in essence, the same principle.

Unlike an intact sperm plasma membrane, which is not permeable to eosin Y, a sperm with damaged cell membranes ("dead" sperm) takes up the red dye and becomes colored (Figure 1D). In the one step eosin-nigrosin stain nigrosin is used to provide the necessary background contrast for the live sperm cells, which remain unstained ("white", Figure 1E) [3]. The eosin-nigrosin solution is prepared by dissolving 1 g of eosin Y and 2 g of nigrosin in a sodium citrate solution. Sperm smears are prepared as described above for morphology using India ink, and the percentage of viable sperm registered. Sperm viability can also be assessed using the LIVE/DEAD sperm viability kit (Molecular Probes), which consists basically of two DNA-binding fluorescent stains: a membrane-permeant stain, SYBR-14, and a conventional dead-cell stain, Propidium Iodide (PI) [5]. This assay is also known generally as the SYBR/PI assay. These stains, while reacting with the same cellular constituent (the nucleic acids), stain cells in a distinct way: SYBR-14 identifies all sperm in the sample (live and dead), exhibiting bright green fluorescence in the nucleus, while PI stains only dead sperm nuclei, which fluoresce bright red. Thus, SYBR-14 penetrates the sperm independently of the membrane integrity status, while PI only stains cells with compromised membrane integrity. Given that PI is more concentrated and that its fluorescence can easily bleed through to the green channel, non-viable sperm appear to be only red using a multiple fluorescence filter set, although, in fact, they contain both dyes. In this assay the sperm suspension ($10x10^6$ sperm/ml) is incubated in appropriate culture medium with the two component kit, at a final concentration of 100 nM and 240 nM for SYBR-14 and PI, respectively, for 10 minutes at 37ºC.

Samples are then mounted in a microscope slide and observed under an epifluorescence microscope. Two major populations of sperm are then observed: living sperm stained bright green (SYBR-14) and dead sperm stained red (PI). In both methods it is also usual to find sperm cells with some degree of coloration at the base of the sperm head, and without color in the reminder regions (eosin-nigrosin); or sperm showing yellow/orange fluorescence instead of clear green or red (PI/SYBR). These sperm cells are usually classified as "moribund" sperm.

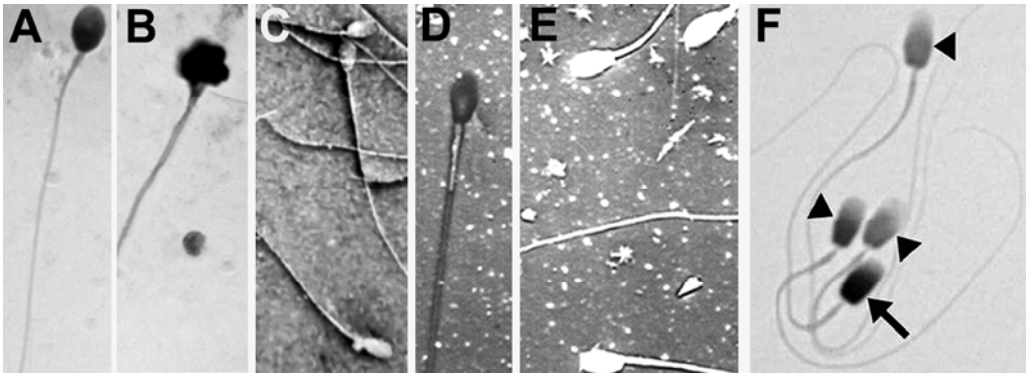

Fig. 1 Optical microscopy methods to analyze mammalian sperm. A) Normal human sperm stained with Diff-Quik; B) Abnormal human sperm head detected with Diff-Quick; C) Equine sperm smear stained with India ink in order to assess morphology; D) Dead equine sperm stained with eosin; E) Live equine sperm unstained with eosin; F) Diff-Quik used to monitor DNA damage in cat sperm (abnormal sperm- arrow; normal sperm- arrowheads). See text for discussion.

3. Acrosome, Capacitation and Membrane status

Other aspects related to mammalian sperm function relate to acrosomal and capacitation status, as only capacitated sperm with an intact acrosome will be able to fertilize an oocyte. Furthermore, some degree of membrane scrambling has also been linked to sperm function. These different variables can be monitored using fluorescence microscopy and appropriate probes.

3.1. Acrosomal status visualized using a fluorescent lectin

The acrosome content marker FITC-PSA (*Pisum sativum* agglutinin linked to fluorescein isothiocyanate) binds to the acrosomal content of sperm from both human and equine species after plasma membrane permeabilization, thus determining the presence or absence of the acrosomal matrix [3]. For equine sperm, stallion semen samples are diluted at a final concentration of 20×10^6 sperm/ml at 35 °C, fixed for 10 min in 2% paraformaldehyde and washed by centrifugation at $600\times g$ for 3 min at 20°C. Samples are then permeabilized using 95% ethanol for 30 min at 4 °C and washed with HH. Afterwards, an incubation period of 15 min at 4 °C with FITC-PSA (Sigma, final concentration of 1 mg/ml) is necessary. Sperm cells samples are then placed on glass microscope slides with coverslips, are evaluated by counting 200 cells/dropt with a fluorescence microscope using a fluorescein filter (i.e "green" fluorescence). Two patterns can be clearly identified: completely apple-green acrosome fluorescence (intact acrosome, Figure 2A, top), and only a fluorescent band at the equatorial segment of the sperm head (reacted acrosome, Figure 2A, bottom). It should be noted that different species may require other lectins to stain acrosomal contents.

3.2. Sperm capacitation and detection of phosphotyrosines

Sperm capacitation involves physiological and functional alterations, such as intracellular calcium increase, cholesterol efflux from the plasma membrane, increase in sperm pH and protein

phosphorylation [6,7]. Protein phosphorylation can be monitored by the detection of phosphotyrosine (PY) residues in sperm, which can be assessed by immunocytochemistry (referred herein as ICC). This technique allows the detection and localization of protein(s) using specific antibodies [8]. First a primary antibody specifically recognizes and binds the antigen to be detected. In order to visualize the protein using epifluorescence microscopy a secondary antibody tagged with a fluorophore, and which is specific for the primary antibody, is then used. Alternatively the primary antibody may be itself fluorescently tagged. ICC protocols involve similar basic steps independently of the protein to be recognized. However, it should be noted that adjustments are certainly necessary in each case (e.g., regarding antibody concentrations and incubation times). In this case human sperm samples (diluted to a concentration of 10 millions of sperm/ml) are attached to coverslips and fixed with 2% (v/v) formaldehyde in phosphate buffered saline (PBS, pH 7.2) for 1 h at room temperature. Cell membranes are permeabilized with PBS contained 1% (v/v) Triton X-100 for 20 min at room temperature. This step will allow the antibody to penetrate into cells, and is only necessary when labeling intracellular antigens. Non-specific antibody reactions can be blocked by a 30 min incubation in PBS containing 1 mg/ml of bovine serum albumin (BSA) and 100 mM of glycine (other authors favor the use of animal serum in this step). Samples are then incubated overnight, at 37ºC, with the primary antibody, in this case a rabbit anti-human phosphotyrosine polyclonal antibody (Zymed, South San Francisco, CA) diluted 1:10 in blocking solution. After washing with 0.1% Triton X-100 (washing solution) for 30 min at room temperature, samples are incubated with the secondary antibody, in this case the Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG; Molecular Probes) diluted 1:200 in blocking solution, for 40 min at 37ºC. Samples are then rinsed in washing solution and mounted in VectaShield mounting medium containing the DNA dye 4,6 diamino-2-phenylindole (DAPI; Vector Labs, Burlingame, CA) and sealed with nail polish. Coverslips are observed under an epifluorescence microscope (fluorescein channel) and 200 sperm are usually counted and analyzed (Figure 2B). This assessment allows the quantification of the percentage of capacitated sperm (sperm with phosphotyrosine staining; Figure 2B).

3.3. Membrane scrambling detected using Merocianine 540

Merocyanine 540 is a useful fluorescent hydrophobic probe for lipid packing because it binds preferentially to membranes with highly disordered lipids. It is also sensitive to heat-induced changes in the organization of membrane lipids, thus allowing the monitoring of alterations in the lipid architecture of the cells [6]. This protocol was optimised for equine sperm cells, and modifications may be necessary for different species. After collection, semen is diluted in 0.5 ml of UHT non-fat milk extender at a final concentration of $25x10^6$ sperm/ml and incubated for 30 min in a water-bath at 35ºC with Merocyanine-540 in the dark (final concentration 2.7 μM). The cells are then observed under fluorescence microscopy in the red channel. For each ejaculate 200 cells should be counted and two sub-populations can be encountered: sperm with instable membranes (positive for Merocianine 540) and non-fluorescent sperm with stable membranes (Figure 2C).

3.4. Membrane integrity detected using the HOS test

The sperm plasma membrane is susceptible to damage caused, for example, by osmotic stress or lipid peroxidation. The physical pressure from osmotic stress results in membrane damage, but, if the limits to membrane integrity were not exceeded, the plasma membrane will respond, behaving as an ideal osmometer. The Hipo-osmotic Swelling (HOS) test is based on this principle. Thus, when samples are places in a hipo-osmotic solution (50 mOsm for equine, 150 mOsm for human), sperm with intact and functional membranes will swell and present typical coiled tails (clearly visible with optical microscopy), contrasting with damaged sperm, were no morphological alterations are detected.

4. Energy production in the midpiece: the activity of sperm mitochondria

Mitochondria are important organelles in sperm homeostasis, and, although some doubts remain as to the true importance of mitochondrially-derived ATP, the activity of sperm mitochondria has been correlated with sperm motility, and thus with fertilization potential. Functioning sperm mitochondria can be monitored using vital fluorescent dyes, such as some MitoTracker family probes or JC1. On the other hand, mitochondrial function is dependent of the presence of several proteins, notably proteins involved in the respiratory chain located in the inner mitochondrial membrane. The presence of these proteins can be detected using standard immunocytochemistry (ICC).

4.1. Assessment of sperm mitochondrial function using MitoTracker

Sperm mitochondrial function can be assessed using fluorescent vital dyes, such as MitoTracker Green (Molecular Probes, Eugene, OR). MitoTracker Green is readily sequestered in mitochondria with a high mitochondrial potential, and the midpiece of sperm with active mitochondria presents green fluorescence, while the midpiece of sperm with non-functional mitochondria remains non-fluorescent [9]. The following protocol describes the use of MitoTracker Green in human sperm mitochondria, and some adjustments may be necessary when applying this procedure in sperm of other species, as well as when using other MitoTracker dyes (e.g, MitoTracker Red, MitoTracker Orange). To stain active mitochondria, a live sperm suspension ($10x10^6$ sperm/ml) is incubated in appropriate culture medium containing 20 nM MitoTracker Green for 30 min at 37ºC. To stain sperm DNA, samples are subsequently incubated with the vital membrane permeable DNA dye Hoechst 33342 (Molecular Probes) diluted 1:1000 in culture medium, for 10 min at 37ºC. Samples are then mounted on a microscope slide and observed under epifluorescence. To determine the percentage of MitoTracker positive sperm, 200 sperm are usually counted per sample, in at least four fields (Figure 2G). This analysis permits the observer to note that human sperm samples are very heterogeneous, presenting both functional and non-functional sperm, as well as to determine the percentage of sperm with active mitochondria in each sample.

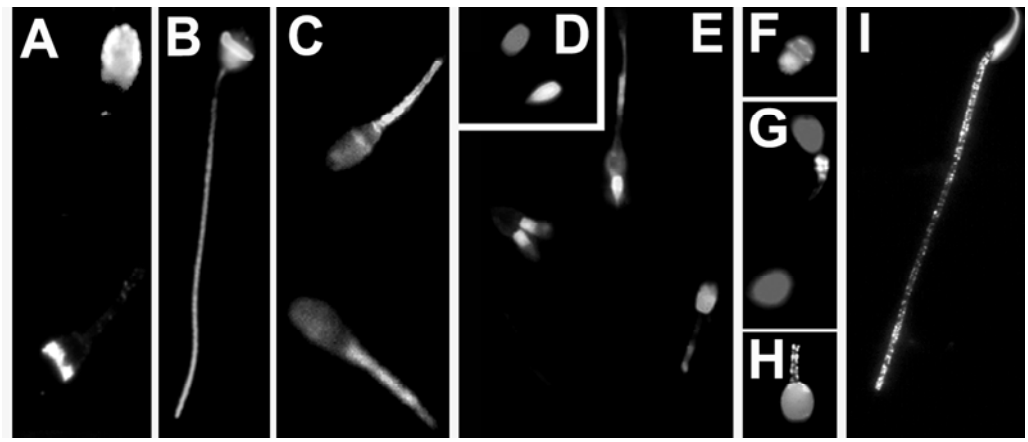

Fig. 2 Fluorescence Microscopy methods to analyze mammalian sperm. A) Equine sperm acrosome stained with PSA-FITC (intact acrosome top, reacted acrosome bottom); B) Putative capacitated human sperm labeled with an anti-phosphotyrosine antibody; C) Equine sperm staining positive for Merocianine 540; D) Cat sperm unstained for Annexin V (only the nucleus is visible); E) Cat sperm positive for Annexin V; F) Human sperm positive for TUNEL, indicating nuclear DNA damage; G) Human sperm positive (top) and negative (bottom) for Mitotracker, indicating distinct mitochondrial activities (the nucleus is also visible); H) Human sperm positive for the mitochondrial protein COX I (the nucleus is also visible); I) Rat sperm mitochondria labeled with JC-1 (the nucleus is also visible). See text for discussion.

4.2. Assessment of sperm mitochondrial function using JC-1

The lipophilic cationic dye JC-1 (5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide), also exhibits potential-dependent accumulation in mitochondria. An increase in mitochondrial membrane potential, and thus an increase in intra-mitochondrial JC-1 accumulation, is indicated by a fluorescence emission shift (from green to red). This happens due to the formation of probe aggregates in highly active mitochondria, with a concomitant shift in fluorescence emission. On the other hand, low membrane potential mitochondria accumulate JC-1 in its monomeric form, and thus exhibit green fluorescence. For JC-1 labeling, sperm cells are incubated with the probe for 10 minutes at 37ºC (final concentration 5.7 nM), and then visualized (Figure 2I). Microscopic evaluation reveals red-orange and green sperm midpieces, and different labeling patterns can be correlated with parameters such as sperm motility [10].

4.3. Detection of mitochondrial proteins using ICC

As in the case with other proteins, ICC coupled to fluorescence microscopy can assess the presence of human sperm mitochondrial proteins. As described above, this technique, which simultaneously allows the detection and localization of the protein(s) in question, uses the specificity of antibodies against the protein(s) to be detected. In this case the protein is the subunit I of Cytochrome C Oxidase (complex IV of the mitochondrial electron transfer chain- COX I) [11] Basically, human sperm are prepared like described in section 3.2. Samples are then incubated overnight, at 37ºC, with mouse anti-human COX I monoclonal antibody (Molecular Probes) in blocking solution. After washing, coverslips are incubated 1h at 37 ºC with appropriate secondary antibodies in blocking solution. After a second wash the coverslips are mounted in Vectashield mounting medium with DAPI, as described before, and observed under epifluorescence. In order to determine the percentage of stained sperm, 200 sperm per coverslip are counted, in at least four fields (Figure 2H).

5. Apoptosis and the status of sperm nuclear DNA: optical and fluorescence-based methods.

The presence of putative apoptotic markers on ejaculated sperm may be of interest in terms of addressing the quality of a sperm sample. Early signs of apoptosis include exposure to the medium of the phospholipid phosphatidytlserine (PS), usually confined to the inner leaflet of the sperm plasma membrane. This process can be monitored using the fluorescence assay involving the PS-binding protein Annexin V [2,4]. More important in terms of possible impairment of sperm function is the presence of chromatin defects in the sperm nucleus, related with either DNA fragmentation or problems in DNA packaging during spermatogenesis. The status of sperm DNA can be evaluated using a variety of assays, which monitor different aspects and have different sensitivities. We describe both a simple optical microscopy test based on the use of the Diff-Quik staining kit, as well as a more complex fluorescence-based assay, the TUNEL assay[2,4].

5.1. Presence of PS on the outer leaflet of the sperm plasma membrane detected using Annexin V

Annexin V is a Ca^{2+}-dependent phospholipid binding-protein that has a high affinity for PS, and can serve as an early apoptosis marker. In this case Annexin V is tagged with the fluorescent moiety Alexa Fluor 568 (red fluorescence, Molecular Probes). Annexin V is usually used together with nuclear stains, which serve as markers for cell permeability, and thus for sperm viability. For example SYTOX green (S), a green-fluorescent dye that is impermeant to live cells, and Hoechst 33342 (H), a cell-permeant stain that emits blue fluorescence when bound to double stranded DNA. Thus Annexin V stains sperm with PS in the outer leaflet of the plasma membrane (a sign of early apoptosis), Hoechst stains the sperm head of all sperm, and SYTOX only the head of non-viable sperm. It is thus possible to distinguish four

sperm subpopulations in a sample: a) viable sperm (A-/S-/H+); (b) early apoptosis (A+/S-/H+); (c) late apoptosis (A+/S+/H+) and (d) necrosis (A-/S+/H+). To perform the assay a sperm suspension (100 µl) in culture medium is incubated with Annexin V (5 µl), SYTOX green (10 µl of a 5 nM solution) and Hoechst 33342 for 15 min at room temperature. The suspension is then centrifuged at 800x g for 10 min, the pellet mounted on a slide with mounting medium, and the sample observed with a fluorescence microscope (Figure 2D,E)

5.2. Assessment of sperm DNA status using the Diff-Quik stain

In the absence of more sophisticated methodology staining kits normally used to assess sperm morphology, such as the Diff-Quik assay (described above), may also be employed to probe the status of sperm DNA. Within the same sample stronger hues of dark label can be seen on the sperm head in some cases (Figure 1F, compare normal sperm (arrowheads) with abnormal staining (arrow)), indicating sperm with abnormal DNA, as shown by comparison performed between the Diff-Quik assay and *bona fide* markers of sperm DNA damage, such as the TUNEL assay [4]. It should be noted that to address the status of sperm nuclear DNA the incubation in the staining solutions must be short. Longer incubation times render all sperm dark and, although still useful to assess morphology, the subtle distinctions between lightly colored and dark colored sperm are lost.

5.3. Assessment of sperm DNA status using the TUNEL assay

The increasing concern regarding the impact of sperm DNA integrity on male fertility has led to the development of several sensitive assays to monitor DNA damage. The terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay is one of the more widely known of these assays. In essence this method transfers a labeled nucleotide (BrdUTP) to the 3'OH group of a damaged DNA strand, a reaction catalyzed by a terminal deoxynucleotidyl transferase (TdT). The modified nucleotide is then detected using an appropriate antibody directly labeled with a fluorescent tag. Sperm with damaged DNA are thus fluorescent. To perform the assay the same fixation, permeabilization and blocking steps, previously described for ICC protocols (see above), are carried out. Human samples are then treated with the APO-BrdU TUNEL assay kit (Molecular Probes). Briefly, samples are washed with the kit washing buffer and then incubated with a DNA labeling solution (10µl of reaction buffer, 0.75 µl of TdT enzyme, 8µl of BrdUTP, and 31.25µl of H_2O) for 1 hour at 37ºC. The cell suspension is then centrifuged and washed in 200µl of rinse buffer and cell pellets are suspended in 100µl of the antibody solution (1:20 of Alexa Fluor 488 anti-BrdUTP antibody diluted in rinse buffer) and incubated for 40 minutes in the dark and at room temperature. Both negative (just the use of secondary antibody-488 anti-BrdUTP) and positive (purposeful induction of DNA damage with H_2O_2) controls should be performed. Stained cells can be detected by fluorescence microscopy on the day of processing, using DAPI (Molecular Probes) as a nuclear counterstain. Samples are placed on coverslips, mounted in VectaShield mounting medium and sealed with nail polish. Sperm cells with nuclear DNA fragmentation stain green (TUNEL-positive cells) while sperm with intact nuclear DNA remain only blue (Figure 2F).

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