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**Running title:** Flow cytometry for sperm evaluation

## Abstract

Flow cytometry is now a recognized methodology within animal spermatology, and has moved from being a research tool to become routine in the assessment of animal semen destined to breeding. The availability of ‘‘bench-top’’ flow cytometers and of newer and versatile markers for cell structure and function had allowed the instrumentation to measure more sperm parameters, from viability to reactiveness when exposed to exogenous stimuli; and to increase our capabilities to sort spermatozoa for potential fertilizing capacity, or chromosomal sex. The present review summarizes the state-of-the-art regarding flow cytometry applied to animal andrology, albeit keeping an open comparative intent. It critically evaluates the present and future capabilities of flow cytometry for the diagnostics of potential fertility and for the development of current reproductive technologies such as sperm freezing, sperm selection and sperm sorting. The flow cytometry methods will probably further revolutionize our understanding of the sperm physiology and their functionality and will undoubtedly extend its application in isolating many uncharacterized features of spermatozoa. However, continuous follow-up of the methods is a necessity owing to technical developments and the complexity of mapping spermatozoa.

Keywords: Animals, Flow cytometry, Spermatozoa, Sperm functionality, Sperm intactness, Semen analysis, Fertility

## Introduction

Evaluation of the spermatozoa of animals has either a purpose of diagnosis of the health of the male reproductive organs or, for those sires destined to breeding, of the odds their semen has to fertilize (or, to lead to fertilization failure, depending on the approach to the problem). During clinical andrological examinations, evaluation of spermatozoa is of paramount importance, since sperm "quality" provides an indication of the normality of testicular function in terms of sperm numbers, their viability (often simply determined by subjective motility judgment) and the normality of morphological features. As well, it provides an idea of the normality of the epididymal function, i.e. sperm maturation, with the display of proper morphological features (displacement of the cytoplasmic droplet from the neck to the annulus) and of activated motility after ejaculation. Fertilizing capacity, which is also reached during epididymal maturation, is not monitored by a simple spermiogramme, and often only assessed after breeding by natural mating or artificial insemination (AI) of a certain number of females, the latter hopefully large enough to keep innate variation at minimum levels. Sperm functionality is, in this regard, obviously modulated or affected by the fluids spermatozoa bathe in, either as seminal plasma (SP) or as extenders used to decrease sperm concentration, or to allow cooling and freezing-thawing of sperm suspensions for AI.

There is a plethora of excellent reviews on this area of sperm evaluation and the reader is advised to consult these[1-14]. Most of these reviews and the literature cited therein consistently concur to a basic fact; routine spermiogramme evaluations are suitable to identify clear-cut cases of infertility and, sometimes, to provide cues for potential sub-fertility, but are not able to prognose levels of fertility of the semen (or the male) in question[7, 13, 15, 16]. The reason why is quite obvious, such assessments simply evaluate basic variables of sperm appearance and viability, but cannot disclose subjacent structural details, biochemical and metabolic details, or the capacity of spermatozoa to interact with the environment, in terms of membrane reactivity, unless a battery of tests is used. Neither can they measure whether the genomic message spermatozoa deliver to the gamete counterpart during fertilization is intact enough to warrant the development of the early embryo. Last, but not least, most routine semen evaluations include small sub-samples (often tested in different assays, so that the same spermatozoon is not evaluated simultaneously by the different tests) of a large, heterogeneous number of spermatozoa in the ejaculate. This implies that the results obtained have an inherent large variability, thus calling for the analyses of larger sperm numbers[13]. It is important to remember that for a given attribute of a cell only a small amount of an attribute might be needed for that cell to function correctly; for another attribute high values might be necessary. Further, it is illogical to assume that all cells of a certain type do their tasks the same way[17].

As already indicated, another major point during semen evaluation is the varying resilience of ejaculated spermatozoa to sustain current and future handling procedures, including extension or re-concentration, cooling, freezing and thawing, high-speed sorting, selection, etc to which ejaculated spermatozoa are nowadays subjected to for use in assisted reproductive technologies, such as artificial insemination or IVF of chilled, frozen-thawed or sexed semen. Markers that can determine how extensive the damages are, and that can monitor their occurrence can be used for a quick, detailed analyses of a large number of spermatozoa of a given sample[10, 18].

Continuous innovation of new fluorescent stains and techniques has facilitated this development. Moreover, with the simplification of the instrumentation, flow cytometry (FC) is now being introduced into handling of semen for AI and even into routine andrological diagnostics.

The present review summarizes the state-of-the-art regarding FC applied to animal andrology, albeit keeping an open comparative intent. It critically evaluates the present and future capabilities of FC for the diagnostics of potential fertility and for the development of current reproductive technologies such as sperm freezing, sperm selection and sperm sorting.

**Principles of flow cytometry (FC) and comparison to other conventional techniques to explore sperm structure and function**

A flow cytometer (also abbreviated FC) is an instrument that can measure physical, as well as multi-color fluorescence properties of particles of cells flowing in a flowing stream[19]. A FC is basically composed of four main systems, fluidics, optics, electronics and software handling, so that the cell suspension flows through a tubular system, it is exposed to laser (or mercury arc lamp in some older instruments) illumination at particular spots and the recorded emissions from the cells as a result of this illumination is digitized and computer-handled to provide understandable results.

A fluid that is moving under a laminar flow specifies the movement and velocities that cells have when passing across the detector. During this transport, cell properties like fluorescence, absorbance, and light scattering can be detected, making use of one or several illumination sources, which might differently excite uploaded markers. In this way, FC detects labeling by multiple fluorochromes associated with individual spermatozoa, so that more than one sperm attribute can be assessed simultaneously, increasing our capacity to correlate these attributes to, for instance, potential fertilizing capacity. Moreover, it makes possible to acquire data from different subpopulations within a sample, thus evaluating heterogeneous populations in different states of activation. Thus the analysis becomes objective, has a high level of experimental repeatability and has the advantage of being able to work with either small or large sample sizes.

Flow cytometers can be broadly classified as analytical (or bench top), sorter and others with miscellaneous functions. Analytical FC are used solely for the analysis of cells or other particles, while FC-sorters have the additional feature that they are able to, based on the results of the analysis, physically isolate the cells or particles of interest. Sorter FC-types can either maintain a continuous fluid or split it into droplets after detector analysis. Fluidic sorters better handle bio-hazardous samples than the droplet sorters, while the latter are used, for instance, to separate spermatozoa by chromosomal sex.

There is a number of commercial FCs developed for specific requirements. These may be broadly function-grouped as:

*1.* ***Analytical***

The most commonly used FCs are sold by Partec (Münster, Germany), Beckman Coulter (Brea, CA, USA), Millipore (Billerica, MA, USA), Accuri (Ann Arbor, MI, USA) and BD Biosciences (Franklin Lakes, NJ, USA).

*2.* ***Sorter type***

Partec (PA-II, PAS-III), Beckman Coulter (MoFlo™ XDP, EPICS® ALTRA™), BD Biosciences (BD FACSAria™, BD FACS Vantage™, BD Influx™) sells sorter type FCs.

*3.* ***Others***

Flow cytometers with new functions are becoming available; Luminex Corporation (Austin, TX, USA) is dedicated to multiple analysis by suspension arrays (the Flexmap3D and Luminex 100/200 instruments), where very small sample volumes can be assayed in a single microplate well. It facilitates nucleic acid assays, receptor-ligand assays, immunoassays and other enzymatic assays. CompuCyte (Beckman Coulter) is a laser scanning solid cytometer which can analyze solid-phase samples together with tissue sections, cancer tissue imprints and cytology smears. This FC is capable to manage high amounts of cell and tissue specimens in a solid substrate analysis. CytoBuoy (Woerden, The Netherlands) is, together with CytoSense, a simplified method (albeit a very expensive one, thus far) to recognize spermatozoa from other cells and debris by shape only. This can, above traditional FC, produce complete signal courses of the measured emission of each particle. It is a portable FC suitable for submerged *in situ* studies up to 200 meters depth together with silico-imaging and video-imaging, mainly applicable for phytoplankton research. The newest BD FACS Array

(BD Biosciences) technology can easily assess cell signaling, cell surface staining and multiplexed bead assay. It is best suited for protein analysis in cell biology, immunology and more specifically for proteomics studies.

## Application of flow cytometry for assessment of sperm attributes

### Enumerating sperm

Total sperm per ejaculate and total sperm per straw are both important. Moreover, with breeding companies decreasing sperm numbers in the AI-doses, accurately accounting how many spermatozoa are inseminated is of utmost importance. The issue of time since collection is also important, since total sperm per ejaculate per hour since latest collection can give information about productivity of the testes, if that time is appropriately short[20]. Total sperm per straw marks that the insemination dose is as advertised and it is likely to maximize fertilizing potential for that bull if little known about his response to decreasing number of sperm/insemination. However, to precisely determine the number of spermatozoa in a suspension volume is difficult, unless time-consuming manual chamber counting is done. Flow cytometry of fluorescence-loaded samples appears as a good alternative to determine sperm total sperm count since FC can discriminate between cells and debris (even considering other cells than spermatozoa) yet counting high cell numbers, thus decreasing variation. For instance, similar results for total sperm count between FC and haemocytometry have been recorded for a number of species [21]. However, the inherent costs of FC have to be considered. Moreover, FC often overestimates the proportion of viable unstained spermatozoa while underestimating that of the damaged subpopulation [22]. We will discuss this phenomenon later in this review. Using fluorescent microbeads, total sperm counts were assessed by FC, simultaneously with other sperm analyses (**Figure 1**, [23, 24, 25]). However, as the beads can introduce another source of variation, alternative FC cell counting methods, like true volumetric counting were developed [26].

### Sperm intactness

#### *- Viability of spermatozoa*

Sperm viability is a convenient, yet imprecise term, because there is no clear demarcation between a living or dead sperm. It is a key determinant of sperm quality and a prerequisite for successful fertilization, becoming more and more important when low sperm numbers are used for AI. Although a motile spermatozoon could be defined as viable, the term "viable spermatozoon" is often linked to an intact plasma membrane, since the plasmalemma is pivotal for sperm interactions with other cells and their environment. Therefore, most viability assay kits just evaluate whether the plasmalemma is intact or not, by using impermeable dyes (those that cannot penetrate an intact membrane) alternatively penetrating dyes (to depict those cells with eroded plasmalemma), or a combination, a matter that shall be described in detail later. Which dye is used can significantly influence the results[27]. Sperm "viability" can be assessed by numerous methods, some manual and other automated, and not only directed to the plasmalemma. Some of them are slow, assess too few spermatozoa, and are thus poorly repeatable. The reader is advised to see the reviews by Rodriguez-Martinez and Barth[11] or Rodriguez-Martinez[13] for a summary of these methods while studying the spermiogramme.

***- Plasma membrane integrity***

The sperm membrane is directly or indirectly related with many sperm functions, warranting the capability of the cell to maintain homeostasis and depict motility and the capacity to interact with the environment, including the lining epithelium of the female genital tract or the oocyte-cumulus cell-complex[7]. Although the sperm plasma membrane covers the entire cell, it consists of several distinct membrane compartments, like the one that covers the outer acrosome membrane, one that covers the post acrosome portion of the sperm head down to the annulus, and lastly another one covering the principal piece and the rest of the tail. The first fluorescent probes applied to evaluate plasma membrane integrity were fluorescein-diacetate (FDA) [28] and carboxyfluorescein-diacetate, CFDA[29]. These dyes are nonfluorescent, but converted to fluorescent derivatives by intracellular esterase enzymes, and trapped by the intact plasma membrane, resulting in a fluorescing cell.

The most commonly used combination of dyes used for membrane integrity checks is SYBR-14/PI (**Figure 2**). In this combined stain, the nuclei of viable spermatozoa fluoresce green while those with eroded plasmalemma are counterstained red, including a moribund cell sub-population (green-red). Propidium iodide is normally used as the viability probe of choice in FC as this supra-vital stain rapidly penetrates non-viable spermatozoa when their plasma membrane is disrupted [30].

This FC–procedure (available as a live/dead kit from Invitrogen (Carlsbad, CA, USA) have been successfully applied for many species, as human[31], bovine[32], porcine[13, 31, 32], ovine[31], lagomorpha[31], murine[31, 33], avian[34, 35], honey bees[36, 37] and fish[38]. Furthermore, it is able to simultaneously evaluate sperm cell viability together with some other attributes *e.g.* in combination with fluorescently labeled plant lectins for simultaneous assessment of plasma membrane and acrosome integrity[39]. The main advantage of this dye combination is that both fluorochromes have the same intra-cellular target (DNA), however it means its main weakness as well: since most DNA is found in the sperm head (and less in the mid-piece in the mitochondria), the assay indicates the integrity of the plasma membrane of the head domain only. Moreover, the probe mentioned above, will not assess the integrity of the plasmalemma covering neither the acrosome, the midpiece nor the rest of the tail.

***- Assessing sperm membrane permeability and stability***

As mentioned above, the intactness of the sperm plasma membrane is of utmost importance for cell integrity and function[40]. Membrane stability and permeability are two closely interlinked functionalities, thus evaluation of membrane permeability elucidates its organization[41], and its capability to undergo biophysical changes such water and the movement of cryoprotectant agents (CPA) during cryopreservation[42, 18]. Cryopreservation causes irreversible damage to the plasma membrane leading to cell death in a large number of spermatozoa[43]. Thus, it is important to know the permeability coefficient of the cells to CPAs, as well as their effect on the hydraulic conductivity of the membrane. Different methods, like Coulter counter, electron microscopy, stopped-flow fluorometry and differential scanning calorimetry have been used for years to evaluate membrane permeability[44]. Differences between cell populations in their uptake of Hoechst 33342 due to altered membrane permeability have also been studied[45]. New fluorescent probes (SNARF-1, YO-PRO-1 and ethidium homodimer) for membrane permeability has improved the value of using FC[42] for this purpose. YO-PRO-1 is an impermeable membrane probe that can leak in after

destabilization of the membrane, as a result of silencing of a multidrug transporter involved in transporting it out of intact cells. Thus, use of YO-PRO-1 is a useful tool for detecting early membrane permeability[46]. The three probes are easily distinguished both in FC and in fluorescence microscopy.

Annexin-V is a calcium dependent probe that has recently being used for tracking externalization of phosphatidylserine (PS) in the sperm membrane. Since PS is normally located exclusively at the inner face of the lipid bilayer, such externalization monitors early changes in membrane stability and intactness, detectable earlier than when PI is used, for instance (**Figure 3**). Combining SNARF-1, YO-PRO-1 and ethidium homodimer offers some advantages over the Annexin-V/PI assay. Whereas in the Annexin-V/PI assay there is always an unstained subpopulation, the triple stain labels all the spermatozoa in the sample. The subpopulation of live cells using the new triple staining concurs with the subpopulation of live cells using the well validated Annexin-V/PI assay. Also, the percentage of early damaged spermatozoa was higher with the Annexin-V/PI assay. This might reflect an increase in membrane permeability, preceding the transposition of PS during an evolving cryodamage. However, it can also be, in a yet to be determined physiological change, a very early step while changes in cell volume regulation and movement of ions occur, during the initiation of apoptosis[47] or cryoinjury[48]. In addition, an earlier inactivation of enzymes involved in maintaining membrane asymmetry than those involved in transporting amphipathic small molecules like YO-PRO-1 might explain this fact.

The FC evaluation of plasma membrane integrity used many fluorescent probes and new probes are emerging rapidly. The Annexin-V/PI assay works for tracking PS-exposure in cryopreserved samples, but due to the presence of cell debris and egg yolk particles, this probe combination might overestimate the unstained sub-population when using FC for detection. Considering this limitation, use of YO-PRO-1 in combination with SNARF-1 and ethidium homodimer has been tested in cryopreserved boar spermatozoa with good correlation with Annexin-V/PI as a result[49]. Another alternative for assessing early changes was to use another triple fluorochrome combination of Merocyanine-540, YO-PRO-1 and Hoechst 33342[50], proving effective for several species[51].

***-Acrosome integrity***

The acrosome is a membrane-enclosed structure covering the anterior part of the sperm nucleus. Powerful hydrolyzing enzymes belong to that structure, which is a basic feature of the sperm head of all mammals[52]. Acrosomal integrity is a prerequisite for fertilization[12], essential for sperm penetration of the zona pellucida (ZP). Biologically, the plasma membrane and the outer acrosome membrane fuse and vesicate during the acrosome reaction when activated by sperm binding to the ZP. Acrosome intactness is traditionally examined *in vitro* using phase-contrast microscopy or differential interference-contrast microscopy on unstained or stained samples for light microscopy[2]. Fluorescent microscopy based staining are chlortetracycline staining (CTC)[53, 54], paramagnetic beads[55], quinacrine[56], isothiocyano-fluoresceinated *Pisum sativum* agglutinin (FITC-PSA)[57] and FITC-concanavalin A (FITC-ConA)[58, 59]. Electron microscopic studies have demonstrated the ultrastructural morphology of acrosomes with the staining patterns of spermatozoa after labeling with the antibodies by Pietrobon *et al.*[60]. Due to high costs and need for trained personnel, such approach is rarely used and suitable only for research purpose. Although epifluorescence microscopy is being

used, Peña *et al.*[61] observed that it was less precise for detecting the percentage of spermatozoa with damaged acrosomes compared to FC.

The FC can advantageously be used instead of epifluorescent microscopy[10], allowing determination of acrosome reaction and its temporal occurrence, and the examination of large sperm numbers. The most commonly used probes are plant lectins labeled with a fluorescent agent (usually FITC), most commonly PSA or PNA. PSA is a lectin from the pea plant that binds to mannose and galactose moieties of the acrosomal matrix. As PSA cannot penetrate an intact acrosomal membrane, only acrosome-reacted or damaged spermatozoa will stain[62]. Arachis hypogaea agglutinin (PNA) is a lectin from the peanut plant is believed to display less non-specific binding to other areas of the spermatozoon, leading some workers to favour this over PSA[63] and it is the lectin of choice when evaluating sperm extended in egg yolk-containing media, as PSA has a nonspecific binding affinity to egg yolk[64] and several non-specific binding sites on the sperm cell surface[63]. Carver-Ward *et al.*[65] proposed that PNA is the most reliable lectin compared to PSA and ConA. Only PNA gives a specific comparison between unreacted and reacted sperm, the differences between the two markers are merely a matter of magnitude. Petrunkina *et al.*[66] observed that FITC-PNA binds to the outer acrosomal membrane[67], so that it can better monitor acrosomal integrity. One of the frequently used fluorochrome combinations for the simultaneous assessment of plasma membrane integrity and acrosomal integrity are FITC-PSA and PI[61]. This double-staining for membrane integrity and acrosomal integrity is relatively reliable for fresh and *in vitro*-capacitated sperm[68]. Problems can arise when frozen-thawed spermatozoa are analyzed. Egg yolk particles from the extender have low fluorescence and, therefore, can be assessed as live acrosome-intact sperm using the PI/FITC-PSA double-labeling method. However, it is yet argued that lectins do not specifically bind to the acrosome region[69] and that other binding sites can be easily distinguished by epifluorescence microscopy, whereas FC only identifies one signal from the entire spermatozoon. A newly developed triple staining (SYBR-14, Phycoerythrin-conjugated PNA and PI, **Figure 4**) procedure was developed and in our hand was found as an efficient method for evaluating acrosomal integrity together with viability[39, 70]. This combination labels every DNA-containing event and the colors of the fluoroprobes fit to the standard filter set of most commercial flow cytometers. The Phycoerythrin-conjugated PNA can also be prepared in the laboratory[71].

***Mitochondrial status***

Mitochondria, located in the sperm midpiece generate a major part of the ATP required for sperm metabolism, membrane function and motility, alongside with anaerobic glycolysis in the cytoplasm[72, 19, 73, 74]. Besides, mitochondria are the coordinators of apoptosis mechanisms in a number of cell systems[75, 76], they are involved in sperm maturation[72] and protection against damage induced by cryopreservation[74]. Recently mitochondrial proteins were found to be involved in the capacitation-dependent tyrosine phosphorylation in spermatozoa[77].

There are approximately 100 mitochondria in the mid-piece of the spermatozoon, depending on the species[78]. Most of these dyes applied in the evaluation of mitochondrial status work by diffusing into living cells and accumulating in mitochondria. The most widely used mitochondrial-specific probe, Rhodamine 123 (R123) is a cationic compound that accumulates in the mitochondria as a function of transmembrane potential[79]. It was historically applied to spermatozoa in combination with ethidium bromide[80]. The R123 accumulates in the mitochondria and fluorescence green, the fluorescence intensity depends

on total amount of functioning mitochondria. Spermatozoa with damaged membranes are usually identified by the uptake of ethidium bromide or PI, to discriminate between those living and dead[80]. Unfortunately this stain does not differentiate between mitochondria that exhibit high respiratory rates. When mitochondrial membrane potential is lost, R123 is washed out of the cells. The main weakness of R123 is that it has low sensitivity and mitochondria have several energy-independent binding sites[81].

The mitochondrial probe MitoTracker Green[82] is nonfluorescent in aqueous solution but fluoresces green upon accumulation in the mitochondria regardless of mitochondrial membrane potential[83]. The mitochondrial stain 5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1, **Figure 5**) that exists as a monomer at low concentration yielding green does permit a distinction to be made between spermatozoa with poorly and highly functional mitochondria[84]. Martinez-Pastor *et al.*[85] observed some relationship between JC-1 staining and motility, although correlation with motility is regulated by many factors. In highly functional mitochondria, the concentration of JC-1 inside the mitochondria increases and the stain forms aggregates that fluoresce orange. When human spermatozoa were divided into high, moderate and low mitochondrial potential groups, based on JC-1 fluorescence, the *in vitro* fertilization rates were higher in the high potential group than in the low potential group[86]. JC-1 has also been used successfully to measure mitochondrial function using fluorometry[87]. The main weakness of JC-1 is that two fluorescent detectors are needed to evaluate one sperm attribute; therefore it is less applicable in multicolor FC experiments. Moreover, Garner and Thomas[84] found the presence of aggregates only marginally detectable after cryopreservation. They hypothesized that cryopreserved sperm cells have damaged metabolic capacity, probably due to glycerol. We developed an alternative labeling technique, using MitoTracker Deep Red[78], which needs only one detector to identify spermatozoa with high and low mitochondrial membrane potential (**Figure 6**). Unfortunately, the spectral properties of this particular dye do not fit to the smaller bench top flow cytometers equipped with a 488nm laser source only.

There are some other mitochondrial stains like 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) and 2-(4-(dimethylamino)styryl)-*N*-ethylpyridinium iodide (DASPEI) which stain mitochondria of living cells, that ought to be tested in andrology. A recent study showed accumulation of cationic lipophilic probe $DiOC_6(3)$ in combination with PI could assess mitochondrial membrane potential effectively in pig spermatozoa[88]. Other studies indicate, however, that $DiOC_6(3)$ is not specific to mitochondrial membrane potential[89].

### *Chromatin intactness*

Evaluation of sperm DNA integrity is of utmost importance owing to early embryo development depending on its normality[12, 13]. Although sperm DNA is packed in a highly compacted and stable form[90], chromatin abnormalities and DNA damage exist, derived from either pre-meiotic testicular insults, during spermiogenesis, when DNA is packed or during further chromatin building (protamine dominance) during epididymal maturation [91-93]. Alternatively, it could be the result of free radical- induced damage[94] or a consequence of apoptosis, in some species[95] Sperm DNA fragmentation can be assessed with terminal transferase dUTP nick-end-labeling (TUNEL) assay, which can identify DNA strand breaks with modified nucleotides. These incorporated labeled nucleotides can be detected in spermatozoa by FC, fluorescence microscopy or light microscopy[96]. TUNEL can simultaneously detect single and double strand breaks. By TUNEL the degree of DNA

damage within a cell cannot be quantified, it only reveals the number of cells within a population with DNA damage An advantage of the TUNEL assay is its application in FC[97], although Dominguez-Fandos *et al.*[98] found 2.6 times greater sperm damage by FC than that of fluorescent microscopy, thus suggesting it may overestimate damage. Chromomycin A3 (CMA3) is a fluorochrome that detects protamine deficiency in loosely packed chromatin, which is correlated to the extent of nicked DNA[10]. The CMA3 is a useful tool to assess the packaging quality of sperm chromatin and allow indirect visualization of protamine deficiency. Traditionally fluorescence microscopy is being used to evaluate DNA integrity by CMA3, but recently it was reported that this CMA3 can evaluate DNA integrity in human spermatozoa though fixation, number of sperm or duration of exposure to CMA3 might affect efficacy of FC outcome[99].

The Sperm Chromatin Structure Assay (SCSA, **Figure 7**) is a technique originally developed by Evenson *et al.*[100] in which the extent of DNA denaturation following acid treatment is determined by measuring the metachromatic shift from green fluorescence to red fluorescence for AO, thus determining the ratio between single stranded and double stranded DNA in each spermatozoon. Consequently the native sperm DNA is stained green while the denatured (either because it was already denatured by the time the acid exposure was issued or broke by the procedure) DNA is stained with orange. In FC, sperm containing greater red to green ratios are exhibiting more DNA denaturation than that of spermatozoa exhibiting lesser red to green ratios[101]. One benefit of SCSA over other methods that evaluate DNA integrity is that the procedure is relatively easy, quick, and thousands of sperm can be evaluated objectively. The SCSA has also been successfully used to check gamete quality after cell manipulation[102]. The most important parameter revealed by SCSA is the DNA fragmentation index (DFI), which assesses the percentage of spermatozoa showing susceptibility of DNA to the acid induced denaturation *in situ*. Unfortunately, the method does not really measure the pure population of spermatozoa really having damaged DNA, which is to be considered a relevant drawback. In humans, and only under certain conditions of assessment of fertility (ART procedures), males with semen samples with more that 30% DFI were considered unable of establishing pregnancy[103]. In animals, where SCSA is the most widespread method to assess chromatin integrity among domestic animals[104-106], particularly related to processed semen (including storage and cryopreservation), there is a relationship between chromatin damage and fertility, but thresholds are far from established with certainty (see Rodriguez-Martinez and Barth[11]). Another parameter indicated by SCSA is the ratio of spermatozoa with high green fluorescence. These spermatozoa have less compacted chromatin and consequently higher green fluorescence and ought to be considered immature[107]. In animals not bred for fertility *eg* horses, there is considerable variation in DFI[106], while the variation is minimal in breeding pigs[108].

## Flow cytometry to assess sperm functionality

### *Changes induced during capacitation*

Capacitation is an important, but rather incompletely understood phenomenon that a spermatozoon undergoes before it can fertilize the oocyte. It primarily takes place in the oviduct[57] when various cellular changes such an increase in membrane fluidity due to lipid modifications, an influx of calcium to the sperm perinuclear and neck regions and flagellum, the generation of controlled amounts of reactive oxygen species, as well as the phosphorylation of protein residues, occur in a concerted manner[109, 110, 111]. Therefore, there is

no general agreement regarding which assay is most appropriate to discern between capacitated and un-capacitated spermatozoa. One of the most widely used method to map these differences is the CTC assay (Chlortetracycline assay) using fluorescence microscopy. This fluorescent antibiotic will detect enhanced fluorescence over the segments of the membrane where $Ca^{2+}$ accumulates, and depict different binding patterns on the sperm head, believed to reflect different stages of the capacitation process[53]. Though CTC is empirically accepted, it is laborious to use and its working mechanism remains still scientifically unexplained.

Early stages of sperm capacitation can be measured by loading spermatozoa with the lipid dye Merocyanine-540[112]. The fluorescence observed by the FC is depending on the degree of lipid disorder[40]. Merocyanine-540 is a hydrophobic dye, which can monitor membrane integrity especially scrambling of phospholipids when coupled with YO-PRO-1 and Hoechst 33342 (**Figure 8,** [50]). Bicarbonate has the potential to increase Merocyanine fluorescence rapidly, which ultimately indicates that phospholipid packing of the plasma membrane becomes disordered by this ion. Likewise, calcium-dependent binding of Annexin-V is able to detect capacitation-related changes in membrane architecture, especially the dislocation of phosphatidylserine (PS) in the lipid membrane leaflet, thus indicating early subtle changes in membrane integrity during capacitation[113]. One study, however, showed that both Merocyanine-540 and Annexin-V indicate early plasma membrane degeneration and not capacitation – at least in human sperm[114].

Calcium influx is crucial for the onset of protein tyrosine phosphorylation. Displacement of calcium in the head plasmalemma occurs during the latter part of capacitation[13]. Indo-1 acetoxymethylester[115] and Fluo[116] are being used for FC determination of calcium. Recently, a FC determination of dynamic quantification of the protein tyrosine phosphorylation in the sperm membrane protein during the capacitation was ascertained[116]. The antiphosphotyrosine antibody can quantify tyrosine phosphorylation in boar spermatozoa using FC, thus waiving the tiresome traditional evaluations with western blotting. The FC will quantitate subpopulations within samples and estimate global phosphorylation in the entire spermatozoon, which might better correlate with the capacitation process, since FC most likely solely evaluates the process in the surface of live spermatozoa[117].

However, when using FC in sperm plasma membrane physiology studies, especially to investigate early membrane changes, one should be careful to avoid introducing labeling artifacts[118].

### *Apoptotic-like changes*

Apoptosis, "programmed cell death", delimits a sequence of events, which leads to the destruction of cells without releasing harmful substances into its surrounding area. Apoptosis plays a distinct role in the development and maintenance of health by eliminating old and unnecessary cells, as well as unhealthy cells. Apoptosis markers have been detected in spermatozoa of many species but the role of these markers are probably not yet elucidated completely[49, 119-122]. Changes of sperm membrane permeability have been considered as a typical event of apoptosis in many studies. Since cryopreservation, freezing and thawing cause cryodamage, and presumably even apoptotic-like changes[123], apoptotic markers can be good tools for forecasting semen freezability and cellular damage occur during cryopreservation, albeit not necessarily indicting apoptosis really occurs[124]. The combination

of Annexin-V with PI is able to simultaneously determine apoptosis-like changes and spermatozoa with compromised plasma membrane. This combination can detect four categories of sperm populations: live, live early "apoptotic", dead and late "apoptotic" and late "necrotic"cells [125]. The APO-BRDU kit to conduct TUNEL assay for determination of DNA fragmentation was found more effective than the Annexin/PI in bull spermatozoa, although detection of necrotic spermatozoa cannot be performed by the APO-BRDU kit. The TUNEL assay measures changes at the later stage while Annexin-V/PI measure early sperm apoptosis-like changes. Poly (ADP-ribose) polymerase (PARP) is a nuclear protein recently being used as an effective apoptosis detector. During apoptosis PARP cleavage occurs which is the classical characteristic of apoptosis[126] , it has been detected in human spermatozoa[127]. The sequence of sperm death might be loss of mitochondrial membrane potential, membrane change (YO-PRO-1+ and PI-) and membrane damage (PI+). Whereas, it was suggested that apoptotic markers like caspase activation and YO-PRO-1 staining might happen only in a specific subpopulation of spermatozoa in red deer[128], and perhaps even reflect methodological deviations.

Caspases, a specific group of cysteine proteases with strong preference for aspartyl residues is considered to be involved in apoptotic degenerative processes as execution agents common to almost all cell types investigated[129]. Still the question is: does apoptosis occur in spermatozoa or only in those species where remnants of cytoplasm are largely available (usually the case for human spermatozoa and for stallion spermatozoa and somewhat for dog spermatozoa, where the amount of cytoplasmic droplets is significant. Nonetheless, Weil *et al.*[130] did not find any evidence for the involvement of caspases in mouse sperm normal death. Further, it was stated that there is no reason of occurring apoptosis in spermatozoa[135]. However, it was evident that caspases were present in the restricted site for apoptosis in spermatozoa, which resulted in poor sperm quality[131].

***Sperm head decondensation during fertilization***

During mammalian spermiogenesis, the sperm chromatin undergoes a step-by-step condensation and packaging mainly characterized by replacement of histones with protamines and the formation of S-S and S-Zn-S bonds between cysteine residues[132], a process that is reinforced during epididymal maturation. Once spermatozoa enter the oocyte at fertilization, its sperm head decondenses to form the male pronucleus, an essential event towards the formation of the zygote[133]. Methods for assessing decondensation must therefore be considered useful for male fertility. Several groups have measured sperm chromatin decondensation by observing changes in size and shape of the sperm head by microscopic examinations when exposed to detergents (mostly sodium dodecylsulphate, SDS) and SS-bond reduction (using dithiothreitol, DTT)[134-136], methods that can predict fertility *in vitro*. Flow cytometry offers an excellent possibility to quantity events associated with chromatin decondensation. Zucker *et al.*[137] detected head decondensation of rat and hamster spermatozoa prior to assessment by increases in light scattering. Samocha-Bone *et al.*[138] evaluated decondensation with the help of AO dye in human spermatozoa. Use of the dye PI with ethylene diamine tetraacetic acid (EDTA) and SDS is able to determine decondensation in boar spermatozoa[139]. Thus, evaluation of decondensation of sperm heads by FC is an emerging but not established technique for assessing male fertilizing potential.

***Detection of oxidative stress and lipid peroxidation***

Oxidative stress may be defined as an imbalance between production of reactive oxygen species (ROS) and the potentiality of antioxidants to scavenge these. A certain level of ROS is essential for sperm functions such as capacitation, acrosome reaction, sperm-oocyte fusion and protein tyrosine phosphorylation[140]. However, it is a matter of concern that high levels of ROS has adverse effect on sperm functionality leading to high rate (20%-40%) of infertility[141]. From studies of different groups it was found that ROS hamper the integrity of plasma membrane and nuclear DNA, leading to breakage of DNA and chromatin cross linking[142]. When the level of these breaks is high, it might lead to lowered fertility[143]. Immature, morphologically abnormal spermatozoa and seminal leukocytes are the main sources of ROS in ejaculates[144]. Numerous direct and indirect methods of ROS determination are available, however, direct *in vivo* determination is difficult as reactive oxidants are very unstable and generally have very short-lived intermediates. Determination of ROS directly *in vivo* might be done by the electron paramagnetic resonance spectroscopy[145] but due to the limitation of temperature and time, indirect methods are preferred. The chemiluminescence assay[146] is one of the widespread used indirect methods of oxidative stress evaluation. And in this system the two main dyes luminol and lucigenin in two different signaling processes are being used. Luminol follows an intracellular deoxygenation reaction, which is mediated by a heterogeneous group of sperm peroxidases, while lucigenin is oxidized at the extracellular level by the superoxide anion[147, 148]. Continuous discovery of new fluorochromes made it possible to analyze oxidative stress by FC. The 2,7-dichlorodihydrofluorescein diacetate ($H_2$DCFDA) is a non-specific dye which could assess intracellular ROS in dog spermatozoa[149]. This dye can be used together with viability dyes for removing the dead spermatozoa[150]. Dihydroethidium (hydroethidine, HE) may be used as a specific probe to detect superoxide anion (**Figure 9**), it is freely permeable and oxidized when bound to DNA, thus becoming valuable to detect superoxide anions in spermatozoa[151], however, other oxygen species might also be detected, depending on the filters used for detection[150]. The MitoSOX™ (Invitrogen) is another new probe able to detect mitochondrial superoxide in the live-cell population of human spermatozoa[152]. The probe is readily oxidized by superoxide, while prevented by superoxide dismutase, thus enabling highly fluorescent oxidative products to be detectable upon binding to nucleic acid. However, most of these probes only measure the amount of lipid peroxidation but cannot locate where this peroxidation occurs. More precise and specific probes (4,5-diaminofluorescein diacetate, BODIPY®, 5-iodoacetamidofluorescein, Invitrogen) to detect oxidative stress in spermatozoa are emerging. Among these, BODIPY® became popular in andrology[153-157]. BODIPY®-based probes have high quantum yield and high sensitivity of fluorescence, and the appearance of BODIPY® labeled spermatozoa can be regarded as an indication of physiologically relevant exposure of phospholipids to ROS[83]. However, it is also assumed that FC-sperm sorting generates lipid pre-oxidation leading to oxidative stress, although there is no direct evidence on this prediction. Prior to FC-sperm sorting, semen samples are highly extended beforehand disturbing the natural defense against oxidation[158]. Levels of 8-hydrodeoxyguanosine have in human spermatozoa been shown to be highly correlated with DNA damage[159, 160].

**Application of flow cytometry for the sorting of spermatozoa by chromosomal sex**

The practical application of sexing spermatozoa, synergistically with other reproductive techniques, could improve the efficiency of animal production both in biological and economic terms. Selection of sex has important implications for populations in which one sex has more intrinsic value (*e.g.* rare animal genetic resources), for instance; stud operations and female dairy replacements, or the avoidance of males for pig production, etc. Sex pre-

selection based on FC works on sperm DNA content measurement to facilitate sorting of X chromosome from the Y chromosome-bearing sperm. The X chromosome carries more DNA than that of the Y chromosome and the principle of FC sex sorting is based on this difference. In FC-based sex sorting, spermatozoa are firstly loaded with a fluorochrome. The non-intercalating vital fluorescent probe Hoechst 33342 is being used in FC based sexing where it penetrates the sperm membrane and binds to the DNA. Droplets containing spermatozoa emitting an appropriate fluorescent signal in the FC acquire electrical charge and are sorted into collection tubes by an electromagnetic field. The development of high speed sorting is one of the major developments of sorting technology, since the unique, asymmetric shape of mammalian spermatozoa makes conventional flow sorters less effective in sexing (**Figure 10**). Garner and Seidel[161] have written a good historical overview. Offspring of pre-determined sex using FC have been successfully produced using fresh and frozen-thawed spermatozoa in several mammalian species: pigs[162], cattle[163], sheep[164] bottlenose dolphins[165], goat[166] and humans[167]. Further refinements to standard FC technology included replacement of the beveled needle by an orientating nozzle with a ceramic tip giving sperm less time to lose orientation, which improved correct orientation of sperm to 70%[168]. Conversion to high-speed modified FC operating under increased pressure had improved the accuracy and efficiency (20 000-25 000 events per second) of sperm sorting and purity of sorted population[169]. Although it was recently reported by some groups that stressful process of sorting might weaken status of livestock spermatozoa, strategies for restoring and strengthen their potentials were also discussed[170]. Besides evaluating sex chromosome content of individual spermatozoa, FC offers a precise tool to do quick cytogenetic analyses on sperm: aneuploidy can be detected based on DNA content of individual sperm cells[171, 172].

**The "damage underestimation" problem**

As it was indicated throughout the present review, one of the biggest challenges in applying FC in spermatology is the correct recognition of spermatozoa and the clear separation from signals of other particles present in the actual samples. With assays like the SYBR14/PI dye combination for viability measurements, it is relatively easy as every sperm cell is labeled with a DNA-specific fluorochrome, while other sperm-sized particles (like lipid droplets) are not. However, in some cases, certain sperm subpopulations remain unlabeled (like in simultaneous viability and acrosome integrity studies with FITC-PNA and PI) and cannot be separated from the signals of non-sperm particles due to their similar scatter properties, resulting in the overestimation of the unlabeled sperm subpopulation and a consequent underestimation of the others. In other cases the applied fluorescent probes may have a non-specific binding to the non-sperm particles – resulting in false positive labeling. The FC seems to overestimate by up to 10% the proportion of viable unstained spermatozoa while underestimating the damaged subpopulation[22]. This, together with the variation that pipetting might introduce when assessing sperm suspensions with low numbers needs further studies.

One approach to solve this "damage underestimation" problem is to use positive labeling on every sperm cell. Thomas *et al.*[64] for example, added SYTO 17, a DNA-specific, membrane permeable dye to the FITC-PNA/PI combination, to add a positive fluorescent signal to the otherwise unlabeled sperm subpopulation with intact plasma membrane and acrosome. Nagy *et al*[39] developed this approach further to use a similar dye combination which can be applied to flow cytometers with a single, 488 nm laser line (the excitation and emission peaks of SYTO 17 are 621 and 634 nm, respectively, therefore it is not suitable for conventional bench-top instruments). In our laboratory, where we use a BD LSR flow cytometer equipped

with three laser lines, we usually apply a DNA-specific, membrane permeable dye to label every sperm cell (as with the exception of pathological cases we do not expect to have other particles with DNA in the sperm samples) and add a fluorescent gate to the scatter gate to narrow our analysis to spermatozoa only, for ex. Merocyanine 540/YO-PRO-1/Hoechst 33342[50] where the Hoechst signal was used as fluorescent gate, or MitoTracker Deep Red 633 in combination with SYBR14, where the later was used to label every DNA-containing event[78]. Obviously, this approach needs a larger, more expensive instrument, and cannot always be followed in laboratories equipped with smaller, single-laser flow cytometers. As an alternative, the use of pretreatments and a mathematical model of alien particles can reduce particle misidentification[173], and can be performed in parallel with the functional assessment in the same FC. Another problem that may arise is that dyes label non-sperm elements, leading instead to overestimation of the population of dead cells[174].

**Future perspectives for flow cytometry in spermatology**

The main direction where the positive power of FC can be further expanded is the development of domain-specific multicolor assays for basic and applied spermatology. Flow cytometry offers the opportunity to measure several sperm attributes on every single spermatozoon and on thousands of spermatozoa per sperm sample, therefore it can improve semen quality control extremely. However, it is important to remember to measure attributes that are different and have low correlation with another. It is important to point out that due to differences in light sources, optical set-up and other adjustable parameters of the instruments, the analysis results at cellular level (the plots) are not easy to compare between laboratories. Thus, there is a need for validating each assay in the users own lab with the use of positive and negative controls. Multiparameter sperm studies by FC are not new: Graham *et al.*[175] measured viability, acrosome integrity and mitochondrial membrane potential simultaneously. Newer fluorochromes with narrower emission spectra and novel flow cytometers with several laser lines widen the possibilities further. We recently started to test a fluorescent probe to evaluate plasma membrane integrity by FC. The probe (Live/Dead Fixable Red Dead Cell Stain Kit, Invitrogen L-23102) has been applied in multiparameter fluorescent microscopy (**Figure 11**, [176]), successfully. It has several advantages over the currently used FC viability assays: the probe labels both the viable and dead cells with the same color, but with different intensity (viable cells seem to be unlabeled when checked with fluorescent microscopy, but the dim fuorescence can be detected with FC). Therefore, only one detector is used for detecting viable and dead spermatozoa. Since the probe labels intracellular amides, it indicates the integrity of the plasma membrane over the whole cell surface, not just over the head domain as does the SYBR14/PI combination. Finally, it is a fixable kit – fixing samples before FC-analyses would offer a more flexible work schedule in the flow laboratory. Our first pilot experiments are promising (**Figure 12**).

Sperm function testing can be expected to shed light on the underlying reasons behind male infertility[177, 178]. It is expected that with the help of new probes a large number of new techniques will be adopted to FC for spermatological analysis. For instance, FC determination of cytosolic calcium, protein tyrosine phosphorylation and cAMP are being developed at our laboratory. To evaluate complex cellular system, multicolor FC can measure intracellular functional markers and cell signaling. Multiplexed bead assays which consists of a series of spectrally discrete particles, defined by either one-color fluorescence intensity or by a two-color fluorescence address, which can be used for capturing and quantitating soluble analytes are suitable for measuring multiple DNA and RNA sequences or multiple proteins.

A new approach of statistical modem-based multi-parametric clustering to identify cell subpopulation has been proposed[179]. This robust clustering method is flexible and gives reproducible results. The novel ImageStream technology is combining FC with cell imaging parallel to quantification and visualization of spermatid specific thioredoxin-3 protein in defective spermatozoa[180]. Cytometry imaging with charge coupled device (CCD) camera facilitate image of sperm while they pass the laser beam. The possibilities of this new FC for sperm evaluation are infinite[181]. The FC methods will probably further revolutionize our understanding of the sperm physiology and their functionality and will undoubtedly extend its application in isolating many uncharacterized features of spermatozoa. Thus, future advancement of FC will continue to evolve as a major tool for spermatology at the single cell level. In **table 1**, we have summarized what we suggest as the best methods for particular properties/functions, as well as some alternative approaches.

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**Competing financial interests**

The authors have no competing financial interests.

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**Figure and table legends**

***Table 1***. A summary of the discussed methods for different endpoints in the review..

***Figure 1***. Color dot plot representing the simultaneous evaluation of viability (SYBR 141/PI staining) and cell concentration. Viable spermatozoa are indicated by green color, dead ones are red, dying spermatozoa are blue. Fluorescent microbeads are indicated by magenta color.

***Figure 2***. Viability evaluation by SYBR 14/PI labelling. 3D contour plot shows distinct peaks for viable and dead spermatozoa, moreover, dying spermatozoa and cell debris can be easily recognized as well.

***Figure 3***. Annexin-V/PI labeling. Density plot shows non-altered spermatozoa (lower left quadrant), PI-negative spermatozoa with exposed PS (lower right quadrant), PI-positive spermatozoa without exposed PS (upper left quadrant) and PI-positive spermatozoa with exposed PS (upper right quadrant).

***Figure 4.*** Simultaneous evaluation of viability and acrosome integrity (SYBR 14/PE-PNA/PI labeling). Color dot plot shows viable, acrosome-intact (lower left quadrant), viable, acrosome-ruptured (lower right quadrant), dead, acrosome intact (upper left quadrant) and dead, acrosome-ruptured spermatozoa (upper right quadrant). Green color indicates viable cells, red indicates dead ones, blue color indicates dying spermatozoa (evaluated as dead).

***Figure 5***. Mitochondrial membrane potential, evaluated by JC-1. Color dot plot shows a raw semen sample before (red) and after (black) freeze-killing (plunging the semen aliquot into liquid nitrogen). List mode files were merged with FCSExpress software (version 3., DeNovo Software).

***Figure 6***. Mitochondrial membrane potential evaluation by Mitotracker Deep Red. Histogram shows spermatozoa with high (marker M1) and low (marker M2) mitochondrial membrane potential.

***Figure 7***. Sperm chromatin analysis by acridine orange staining. Intact spermatozoa are black, cells with increased amount of single-stranded DNA (indicating DNA damage) are red, spermatozoa with immature chromatin are indicated with green color. Cell debris is indicated by orange color and is gated out during analysis.

***Figure 8***. Plasma membrane asymmetry in a bull sperm sample measured by Merocyanin 540/YO-PRO-1 labelling. Contour plot shows viable cells with stable plasma membrane (lower left quadrant), viable cells with destabilized plasma membrane (lower right quadrant), and dead spermatozoa (upper left and right quadrants).

***Figure 9***. ROS analysis by Hydroethidine and Hoechst 33258. Cells in the lower left quadrant are membrane-intact, negative for ROS-production. Cells in the lower-right quadrant are membrane-intact, positive for ROS-production. Cells in the upper-right quadrant have a damaged membrane and are positive for ROS-production.

***Figure 10***. Separation of X- and Y-bearing spermatozoa with a conventional sorter flow cytometer, without orienting head. The majority of spermatozoa are not properly oriented,

therefore excluded from analysis (sperm events outside of the yellow rectangular region on the left contour plot). Spermatozoa in the yellow region are gated to the histogram on the right.

***Figure 11***. Merged fluorescent and DIC photos of bull spermatozoa labeled with Live/Dead Fixable Red Dead Cell Stain Kit (Invitrogen L-23102) and Alexa Fluor 488 PNA (L21409, green). Spermatozoa with intact plasma membrane and intact acrosome show no fluorescence, cells with disrupted plasma membrane show red tail and/or head, and ruptured acrosomes are green.

***Figure 12.*** Contour plot shows spermatozoa labeled with Live/Dead Fixable Red Dead Cell Stain Kit (Invitrogen L-23102). Intact cells are in lower left quadrant, dead spermatozoa are in lower right quadrant.

Table 1

Table title: Summary of sperm attributes and possible methods for evaluation

| Sperm attribute | Possible methods |
|---|---|
| Enumeration of spermatozoa | True volumetric counting, fluorescent staining |
| Plasma membrane integrity | SYBR-14/PI, CFDA |
| Plasma membrane permeability and stability | Annexin V/PI , Hoechst 33342, YO-PRO-1, Merocyanine 540, SNARF-1,Ethidium homodimer |
| Acrosome integrity | SYBR-14/PE-PNA/PI, FITC-PNA/PI, FITC-PSA/PI |
| Mitochondrial status | JC-1, Mitotracker Green, Mitotracker Deep Red |
| Chromatin Intactness | SCSA, TUNEL, CMA3 |
| Apoptotic-like changes | Annexin V/PI, YO-PRO-1 |
| Oxidative stress | $H_2$DCFDA, HE, MITOSOX™, BODIPY® probes |
| | |
| | |
| | |

Abbreviations: CFDA: carboxyfluorescein diacetate, PI: propidium iodide, SNARF-1: seminaphtorhodafluor-1, PE: phycoerythrin, PNA: peanut agglutinin, FITC: fluorescein diacetate, PSA: pisum sativum agglutinin, JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, SCSA: sperm chromatin structure assay, TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling, CMA3: chromomycin A3, $H_2$DCFDA: 2',7'-dichlorodihydrofluorescein diacetate, HE: hydroethidine

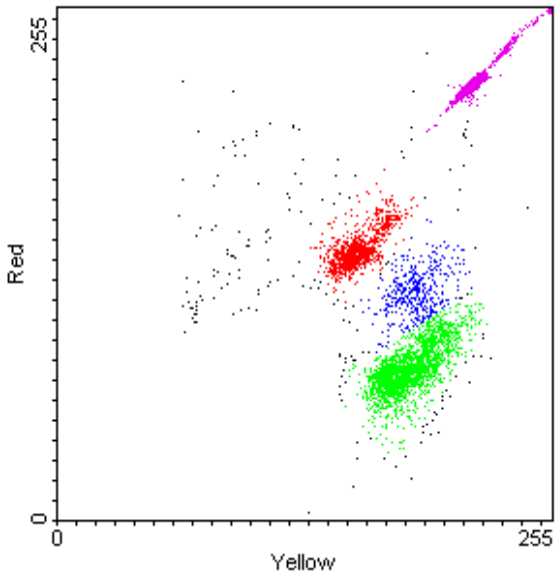


Fig. 1.

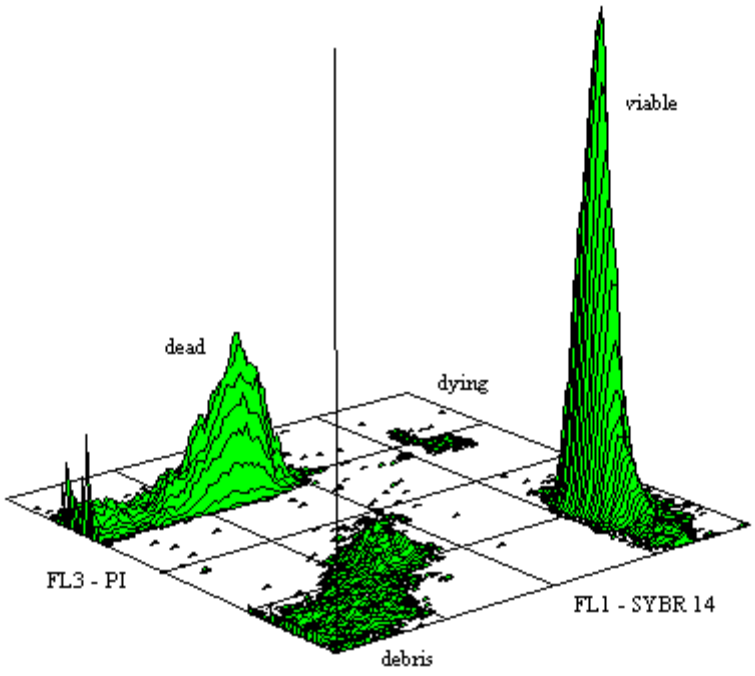


Fig. 2.

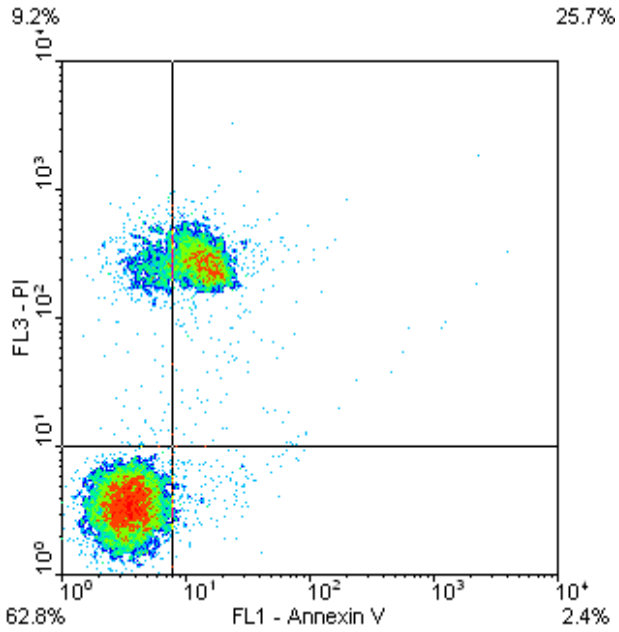


Fig. 3.

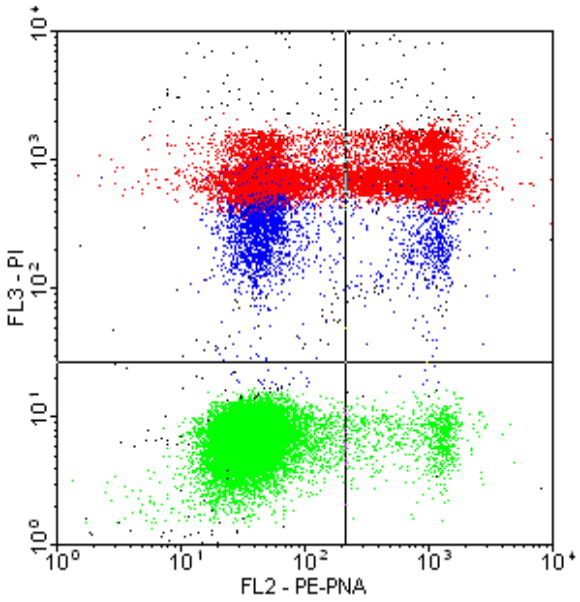


Fig. 4.

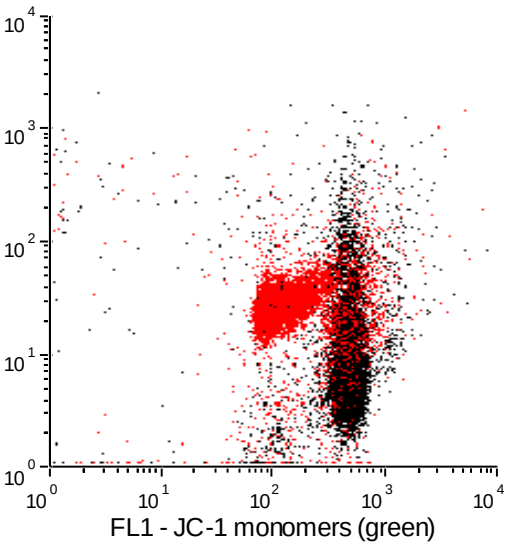


Fig. 5.

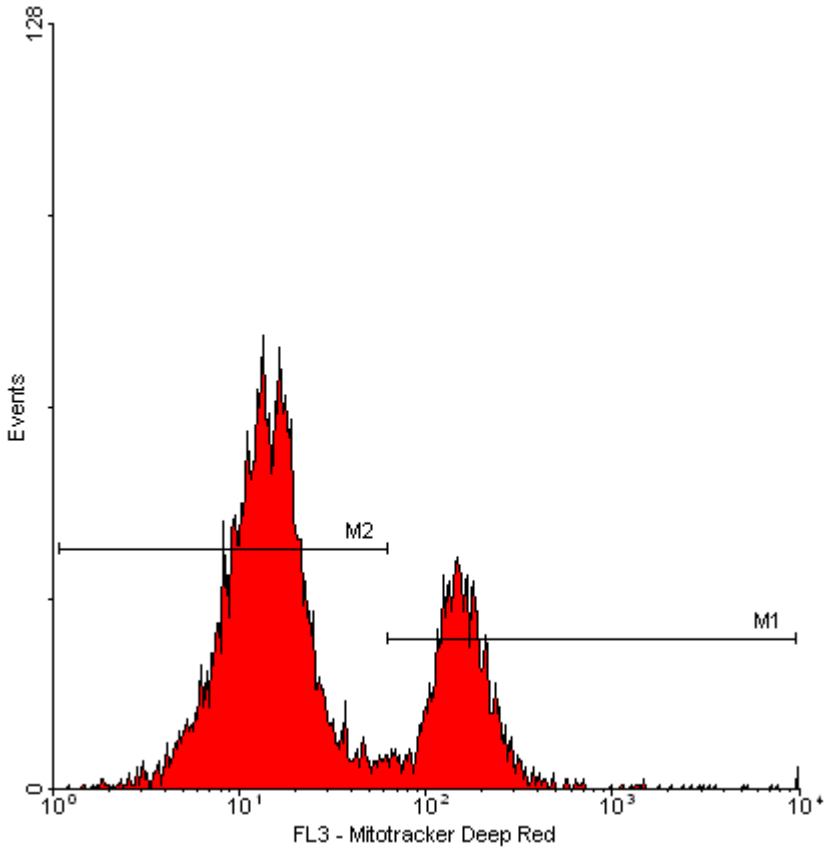
128
Events
0
M2
M1
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
FL3 - Mitotracker Deep Red

Fig. 6.

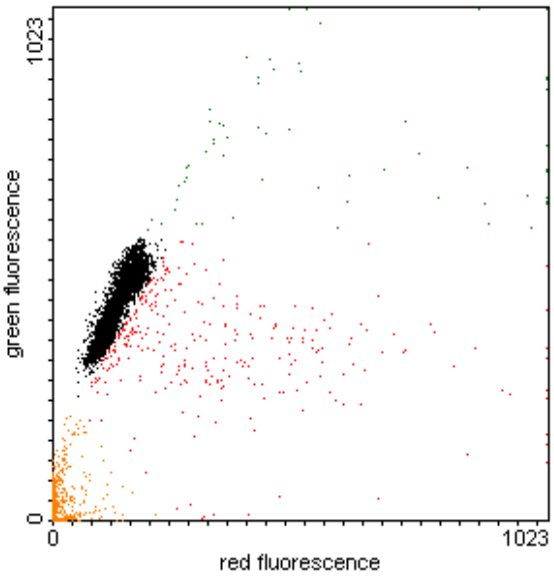


Fig. 7.

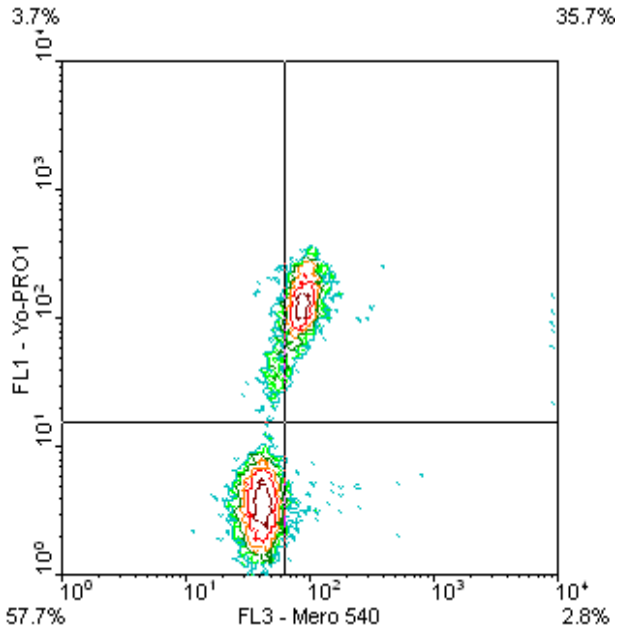


Fig. 8.

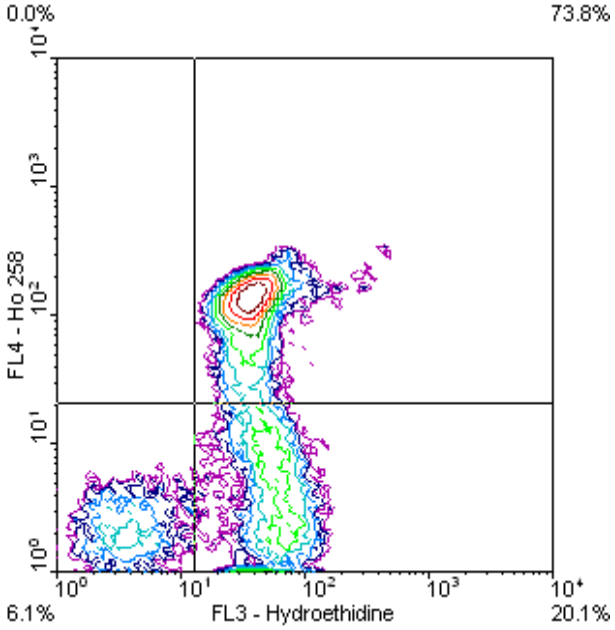


Fig. 9.

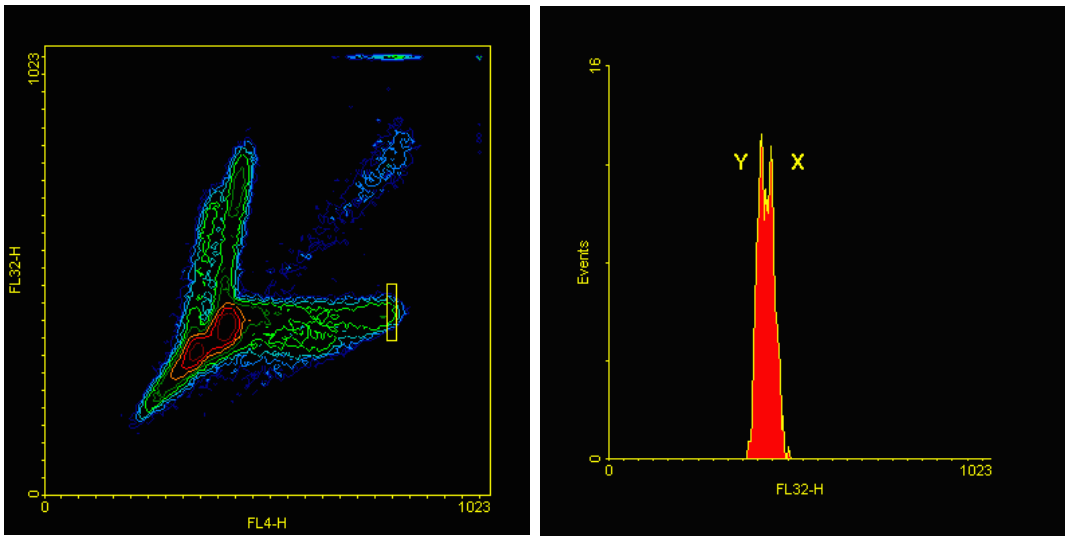


Fig. 10.

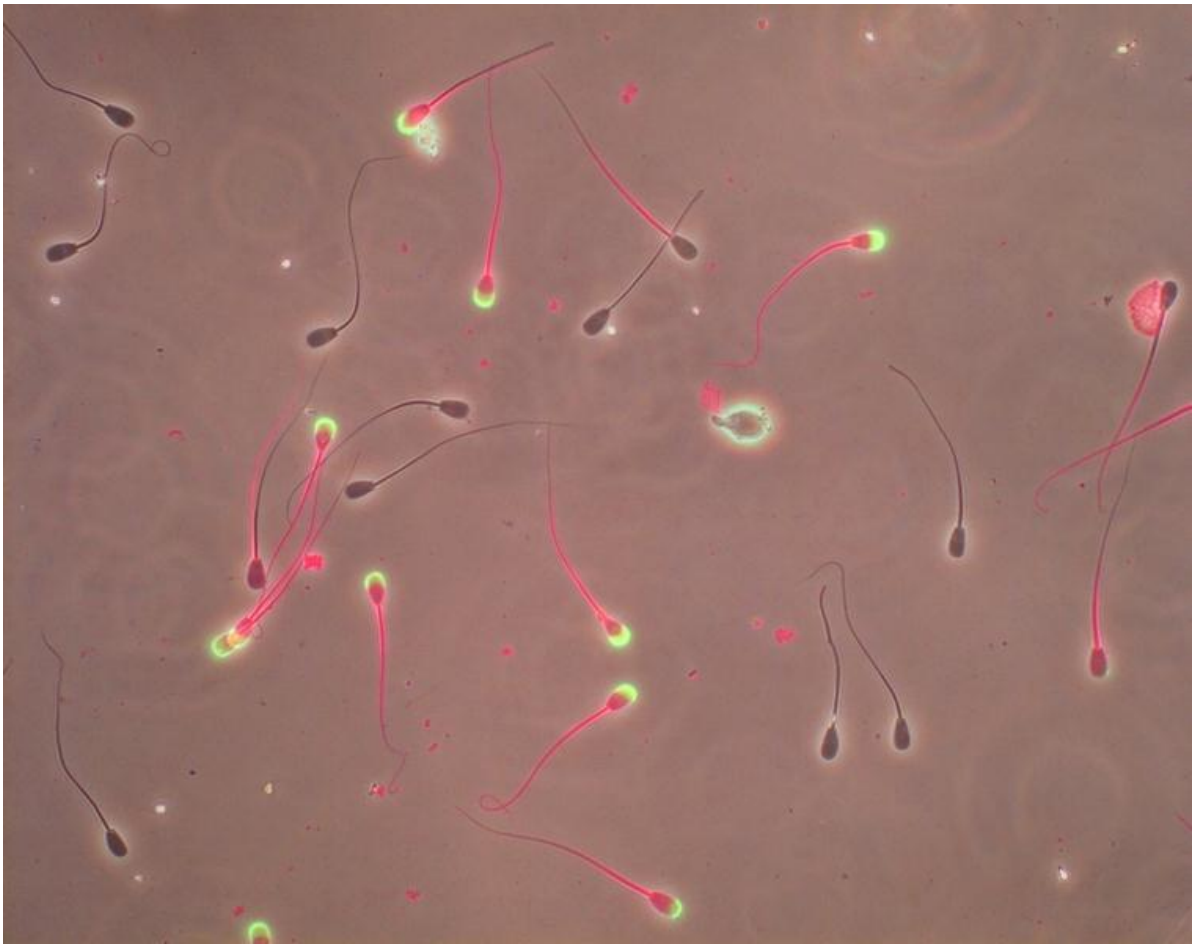

Fig. 11.

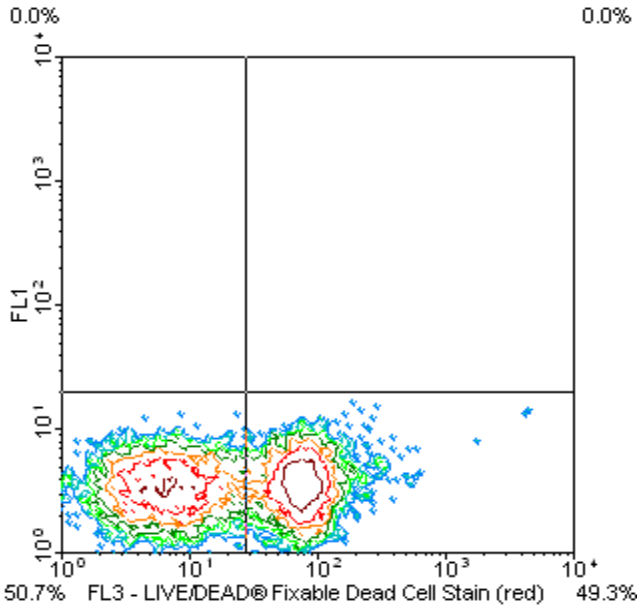
0.0%
0.0%
FL1
10⁴
10³
10²
10¹
10⁰
10⁰
10¹
10²
10³
10⁴
50.7%
FL3 - LIVE/DEAD® Fixable Dead Cell Stain (red)
49.3%

Fig. 12.