Determination of Mitochondrial Function in Sperm Cells

13

N. Srivastava and Megha Pande

Abstract

With the advancement of science, detecting functionality of mitochondria has become one of the desirable parameters to evaluate sperm quality. This is further aided by availability of wide spectral range of fluorescent probes that has advantage of simultaneous multi-parametric assays. Apart from application of fluorescent probes, computer-assisted image analyzers can be used to assess mitochondrial functionality via motility attributes. In this chapter, we have attempted to describe evaluation of mitochondrial functional status using fluorescent dyes and thus have listed relative merits, protocols, and what to look for in the stained sperm sample. This important chapter also includes triple staining of sperm cells to elucidate integrity of acrosome, plasma membrane, and mitochondrial functions simultaneously. Additionally, procedure for flow cytometry of fluorescent-stained sperm cells as an objective method and tetrazolium (MTT) reduction assay as an easy, inexpensive, and rapid spectrophotometric protocol to determine mitochondrial function in spermatozoa is described.

Keywords

Mitochondria • Membrane potential • Sperm • Fluorescent probes • Potentiometric dye

Contents

N. Srivastava (✉)
Quality Control, Semen Freezing Laboratory,
ICAR-Central Institute for Research on Cattle,
Meerut 250001, Uttar Pradesh, India
e-mail: sangnee15@gmail.com

M. Pande
Animal Physiology and Embryo Transfer Laboratory,
ICAR-Central Institute for Research on Cattle,
Meerut 250001, Uttar Pradesh, India

N. Srivastava, M. Pande (eds.), *Protocols in Semen Biology (Comparing Assays)*,
DOI 10.1007/978-981-10-5200-2_13

13.1 Introduction

Evaluation of sperm damages has always been one of the hotspots in the field of assisted reproductive technologies (ART). Earlier, optical microscope was the principle instrument to evaluate sperm damages by conventional parameters, e.g., viability, motility, intactness of the membranes, and morphological studies. With the advancement of science, it is now possible to examine integrity of various organelles like acrosome or mitochondria.

The mitochondrion, also known as powerhouse of the cell, is the key indicator of the normal cellular function, for they are the principle producers of ATP (for energy) and that of free radicals as well as for ion homeostasis. The sperm mitochondria (approximately 50–75), localized in the mid-piece of the flagellum, contain one copy of mitochondrial DNA (mtDNA) in each [1]. The mtDNA wraps around the outer dense fiber axoneme complex helically in a species-specific manner during sperm synthesis to form a cylinder-shaped mitochondrial sheath. Inside this sheath, adjacent mitochondria associate both end to end and along their lateral surfaces (concentrated array of mitochondria adjacent to the flagellum) provide some of the energy for sperm motility [2] and to maintain cellular integrity. In contrast, investigations have shown that energy-dependent tubulin sliding, very much required for sperm motility, takes place at the faraway place (distal) in the flagellum, indicating that energy consumption is rather faraway from the site of aerobic ATP production [3]. Nevertheless, mitochondria provide the midpiece and the sperm head with ATP required for motility and housekeeping processes of membranes; the most important and ATP-consuming housekeeping process is maintenance of Na+/K+ gradient, thus indirectly driving other transporters to regulate the chemical and electric gradient over the plasma membranes. The process of maintaining gradient also indirectly drives other transporters and thus regulates the chemical and electric gradient over the plasma membrane. It is interesting to note that fresh sperm largely produces ATP by glycolysis (>90%) even in medium containing oxygen [4]. It is thus amply evident that for sperm to survive in female reproductive tract and during ART, functional integrity of the mitochondria is of utmost importance. Although paternal mitochondria might enter the oocyte, they are destroyed shortly after fertilization.

In fluorescent microscopic evaluation, functionality of mitochondrion is detected by organelle-specific dyes. These dyes generally exploit the membrane-polarizing attribute of mitochondria to differentiate metabolically active from that of functionally dead spermatozoa. Fluorescent probes that detect mitochondrial membrane potential (MMP) are always positively charged, causing them to accumulate in the electronegative interior of the mitochondrion (Fig. 13.1). This change in the mitochondrial membrane potential due to apoptosis or other events is measured by a variety of fluorescent techniques such as flow cytometry and fluorescent imaging. Investigators have used different fluorescent dyes, viz., Rhodamine 123, tetramethylrosamine (conventional dyes), MitoTracker® (a series of patented probes specific to mitochondria, Molecular Probes, Eugene, Oregon, USA), and others (Table 13.1), and reported their utility in evaluation of mitochondrial activity. Structural probes are capable of staining mitochondria regardless of their membrane polarization. Mitochondrion-selective probes allow investigators to evaluate their activity, localization, and relative abundance in addition to monitor the effects of some drugs on mitochondrial activity, such as anesthetics (in human medicine).

Though electron microscopy (2D and 3D) has evolved proving deeper insight into mitochondrial function, being a highly specialized field, we have not delved into this aspect here. This chapter describes relative merit of probes and procedures involved in determining mitochondrial membrane potential in a sperm cell.

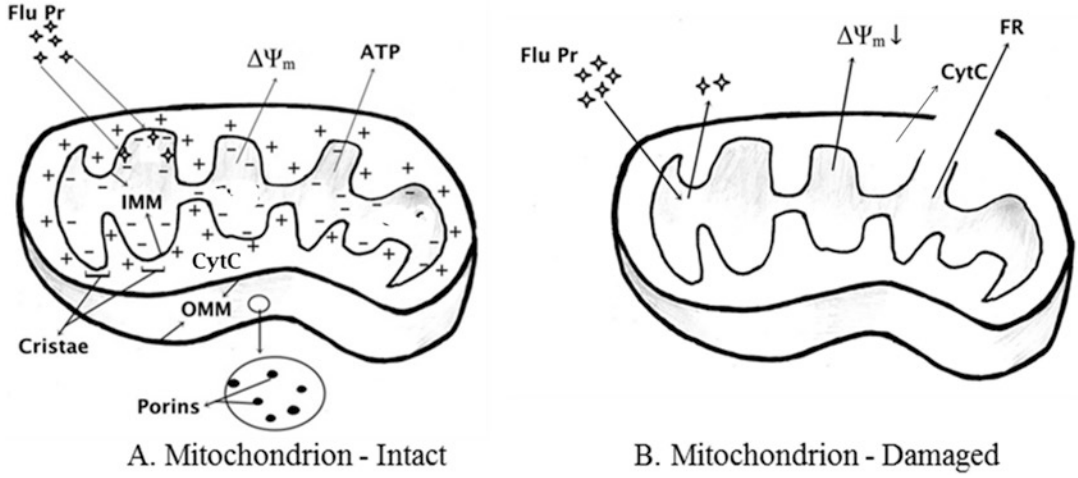

Fig. 13.1 Evaluation of the functional state of spermatozoa mitochondrion. Development of proton gradient to facilitate the electron transfer to O_2 to form water in mitochondrion results in generation of ATP by the enzyme ATP synthase. This development of mitochondrial membrane potential (ΔΨm) of the inner mitochondrial membrane (IMM) can be evaluated by the potentiometric probes (fluorescent probes, Flu Pr) like JC-1. This probe diffuses over the sperm plasma membrane and the outer mitochondrial membrane (OMM), accumulating in the IMM. While several of the potentiometric probes only fluoresce when intercalated in the IMM, other probes fluoresce after interacting with free radicals generated by mitochondrion that convert them to fluorescence. Because of their negative membrane potential, mitochondria accumulate lipophilic cations. Once mitochondrion loses its integrity, the mitochondrial membrane proton gradient is lost, and cytochrome C (CytC) required for electron transport chain diffuses out. With the outflow of CytC and free radicals (FR) from mitochondria, respiratory activity of mitochondrion ceases, and potentiometric probes do not recognize IMM and no fluorescence is generated

Table 13.1 Fluorescent probes used for evaluating mitochondrial attributes

MMP-sensitive (functional) probes			MMP-insensitive (structural) probes	
Rhodamine dyes	Carbocyanine dyes	Rosamine dyes	Cardiolipin binder	Thiol (cysteine residue) binder
Rhodamine 123	JC-1	MitoTracker® Orange CM-TMRos/CM-H_2TMRos	NAO	MitoTracker® Green FM
TMRE	JC-9	MitoTracker® Red CMXRos/ CM-H_2TMRos	Mito-ID	MitoTracker® Deep Red 633
TMRM	$DiOC_6(3)$	Infrared fluorescent MitoTracker®		MitoTracker® Red 580

TMRE tetramethylrhodamine ethyl ester, *TMRM* tetramethylrhodamine methyl ester, *NAO* 10-*N*-nonyl acridine orange

13.2 Relative Merits of Fluorescent Probes Used for Detecting Mitochondrial Activity

Rhodamine 123 (R123) was the original fluorescent probe to specifically stain functionally active mitochondria. Rhodamine 123 characteristically fluoresces red following development of proton gradient over the inner mitochondrial membrane (IMM). Conventional dyes such as tetramethylrosamine and R123, although readily taken up by functional mitochondria, have disadvantage of leaching out of the sperm cells following loss of the membrane potential, thus limiting their application in investigations involving application of aldehyde-based fixative or other probes that influence the energetic state of the mitochondria.

Moreover, results and interpretation of mitochondrial function studied in their native (live) state differ greatly from that of investigations carried out either on mechanically isolated mitochondria or detergent-permeabilized cells. This is due to the role played by cytosol as external medium on the mitochondrial function. To overcome the problem, the fluorescent probes (MitoTracker®, Molecular Probes, Eugene, Oregon, USA) were developed. Using MitoTracker® dyes, stained sperm suspensions can be fixed with mitochondria retaining the stains. They selectively label the respirating mitochondria in live cells and, therefore, are suitable for multi-parametric sperm assessments [5]. Reduced MitoTracker® CMTMRos and CMXRos (also called MitoTracker® Orange and MitoTracker® Red, respectively) belong to the rosamine molecule family. These fluorescent probes have twin advantage of being retained by the sperm even after fixation with formaldehyde and red/orange fluorescence suitable for multi-parametric studies. However, MitoTracker® Orange CMH2TMRos and MitoTracker® X-Rosamine CM-H2XRos fluorescence fluoresce characteristically after oxidation (takes place only under oxidative respiration in live cells), making them suitable for discriminating between apoptotic cells from that of aerobically capable living sperm [6].

Another probe, MitoTracker® Red CMXRos, was used to investigate and classify cryo-damaged sperm cells into structurally normal, mid-piece droplet, mid-piece or flagellar droplet, head abnormalities, damaged mid-piece or flagellum, and spermatids [7]. On the other hand, some of the fluorescent probes like JC-1 change their fluorescent properties depending on the changes in the potential of the IMM. Thus, orange fluorescence of the JC-1-stained sperm depicts functional mid-piece, whereas switching of fluorescence from orange to green shows IMM depolarization [8] and thus can be used to report depolarization of the IMM as an indicator of mitochondrial functionality. Smiley et al. [9] have suggested JC-1 as the most appropriate choice for assessing mitochondrial membrane potential. In support of above observation, while evaluating various mitochondrion-specific dyes, Garner et al. [10] found the differential staining of JC-1 to provide a more rigorous estimate of metabolic function than R123 or MitoTracker®. DiOC6(3), a carbocyanine dye from the DiOC family, cannot be used exclusively to stain MMP measurement in intact cells, except after dissipating the plasmatic and ER membrane potentials. Investigators are advised to monitor the dye and cell concentration with care to produce rigorous and reproducible results. When probes such as DiOC6(3) are employed at low volume (10–20 nM), it quickly attains equilibrium in the mitochondrion with low quenching effects. On the other hand, the use of higher concentrations (more than 50 nM) may result in staining of structures other than mitochondrion (plasma membrane and endoplasmic reticulum) and in fluorescence quenching [11]. (Refer to Table 13.2 for relative merits of fluorescent probes.)

13.3 Microscopy in Mitochondrial Evaluation

In evaluating mitochondrial function, cytometric techniques such as flow cytometry and confocal microscopy offer several advantages. The former technique has the benefit of measuring fluorescence associated with single particles and is effectively used even with low amount of biological materials or probe. This confers advantage of limiting quenching and light-scattering effects. In flow cytometry molecules that are not incorporated into cells or organelles are not analyzed, thus increasing accuracy of the result. Additionally, this technique allows for generation of quantitative data by assessing morphological and functional characteristics of whole spermatozoa or mitochondria and helps in sorting of highly purified sperm population in which mitochondria can be studied. On the other hand, microscopy involving fluorescent or confocal microscopes provides accurate images of even the single intracellular mitochondria and permits the observation on their distribution and organization as reticular network in most of the cell types [11]. Compared to flow cytometry, confocal microscopy

Table 13.2 Relative merits and demerits of mitochondrion-specific fluorescent probes

Fluorochrome; excitation/emission spectra ($\lambda_{ex}/\lambda_{em}$)	Advantage(s)	Disadvantage(s)
Rhodamine 123; potentiometric probe (488/515)	Rapid cellular uptake and equilibration [12]	Not well retained in cells after washing[14]
	Can be combined with PI for viability study [13]	Quenching phenomenon when used in high concentration [15]
	Highly specific for living cells[8]	Nonspecific binding to mitochondria[16]
		Does not discriminate between low and high MMP
		Least photostable, spectral overlap with other (green vs. red) probes [8]
		Intermediate inhibition of mitochondrial respiration [14]
MitoTracker® Green FM; carbocyanine-based probes (488/515)	More photostable than R123 [14]	Not specific for mitochondria with differential MMP [17]
	Commonly used for mitochondrial localization, size, and structure measurement [17]	
	Multi-parametric sperm assessments [5]	
MitoTracker® Red CMXRos; derivative of X-rosamine (568/598)	Highly sensitive [14]	Excitation wavelength may not be suitable for benchtop flow cytometer
	Multi-parametric sperm [5]	
	Can be used to classify damages to sperm morphology[7]	Requires more sophisticated equipments [18]
	Differentiates aerobically capable sperm from deteriorating mitochondria [6]	
MitoTracker® Deep Red 633; carbocyanine-based dye (628/650)	Highly sensitiveand can be used prior to fixation [14]	Overlapping photo-spectra may interfere in multi-parametric staining
	Multi-parametric sperm assessments [5]	
MitoTracker® Orange,derivative of tetramethylrosamine (488/550)	Can be used prior to fixation [4]	Excitation wavelength may not be suitable for benchtop flow cytometer
	Multi-parametric sperm assessments [5]	Requires more sophisticated equipments [18]
MitoTracker® Orange CM-H_2TMRos;reduced probes (551/576)	Highly sensitive [14]	Excitation wavelength may not be suitable for benchtop flow cytometer
	Differentiates aerobically capable sperm from deteriorating mitochondria [6]	Requires more sophisticated equipments [18]
NAO; 10-*N*-acridine orange (495/519)	Well retained in the mitochondria [19]	Toxic at high concentration[21]
	Uptake does not depend on MMP [20]	
Mito-ID Red, (558/690)	Aldehyde, binds cardiolipin,F	Least cytotoxic and phototoxic, superior photostable [14]
JC-1; carbocyanine group (488/535) (monomer, green), 590 (J-aggregates, orange)	Dual fluorescence (orange and green) to differentiate sperm with varying MMP	JC-1 red fluorescence of aggregates can be sensitive to photo-bleaching [14]
	Can be used for both quantitative and qualitative analyses[8]	Slow probe [8]
	Can report depolarization and functionality of mitochondria [8]	
	Better than R123 or MitoTracker® dyes in evaluating mitochondrial activity [10]	
	More specific for mitochondrial vs. plasma membrane potential[22]. More consistent in response to depolarization than R123/$DiOD_6$(3) [5]	
	Can be used to stain intact tissue or isolated mitochondria [22]	

(continued)

Table 13.2 (continued)

Fluorochrome; excitation/emission spectra ($\lambda_{ex}/\lambda_{em}$)	Advantage(s)	Disadvantage(s)
TMRM/TMRE; rhodamine derivatives (548/573, 549/574, resp.)	Nontoxic, do not form aggregates, do not show binding-dependent fluorescence [23]	Minimum concentration of probe is to be maintained for repeatable results
	Low concentration is sufficient for staining [24]	Quantitatively related to the contrast between intra- and extracellular
	Specific for mitochondria not other organelles [24]	fluorescences that must be taken into account during the MMP measurement in intact cells [14]
	No inhibition of mitochondrial respiration by TMRM, intermediate by TMRE [14]	
$DiOC_6(3)$; carbocyanine dye (484/501)	Can be combined with PI for viability study [25]	Not specific to mitochondria, stains Golgi apparatus at high concentration [14]
		Does not discriminate between low and high MMP [8]
		Strongest inhibitor of mitochondrial respiration [14]
MTT; tetrazolium redox salt (protocol)	Simple, rapid, and reliable [26]	Not suitable for cytohistochemistry procedures which require finer study of cellular structures
	Inexpensive [27]	

PI propidium iodide, *MMP* mitochondrial membrane potential, *TMRE* tetramethylrosamine ethyl ester, *TMRM* tetramethylrosamine methyl, *NAO* 10-*N*-nonyl acridine orange

measures only a few sperm cells at a given time and is unsuitable to evaluate large homogenous sperm cell population. On the other hand, compared to conventional fluorescence microscopy, confocal laser scanning microscopy offers several advantages. For one, the blur due to out-of-focus images is nonexistent, and 3D reconstructions from serial sections (even in live cells) provide detailed analysis of the samples [28]. On the other side of the spectrum, there are several kits available for mitochondrial evaluation. Such kits are fast and simple, offer a convenient labeling and assaying mitochondrion, and are complete with required reagents for flow cytometry or fluorescent microscopy. Limitation is that most of the kits are often evaluated on a limited number of cells. Though kits are characterized by a short sampling time, they are expensive in comparison to laboratory-made reagents. It is therefore necessary to verify their specificity on sperm cells [14].

13.4 Color Palette for Mitochondrion in Simultaneous Multi-parametric Staining

In many of the investigations, evaluation of more than one parameter is required to be studied simultaneously. In such cases, an investigator is required to carefully select color combination of various fluorescent probes for each parameter to arrive at unambiguous results. Therefore, keeping in mind the experimental need, a table depicting color palette of some of the fluorescent probes used for evaluating mitochondrial function is given below (Table 13.4). For example, depending upon the fluorescence color of the mitochondrial probe given below, viability probes such as propidium iodide, SYBR-14, or Hoechst 33342 (red, green, or blue, respectively) can be selected (Table 13.3).

Table 13.3 Fluorescent palette of probes used for evaluating mitochondrial function in multi-parametric study of sperm quality

Red fluorescence	Yellow and orange fluorescence	Green fluorescence
MitoTracker® Red CMXRos	MitoTracker® Orange CMTMRos	$DiOC_6(3)$
MitoTracker® Red CM-H_2XRos	Rhodamine 6G	Rhodamine 123
MitoTracker® Red FM	Tetramethylrosamine	10-*N*-nonyl acridine orange
JC-1/JC-9	Tetramethylrhodamine methyl ester (TMRM)	JC-1/JC-9
MitoTracker® Deep Red FM	Tetramethylrhodamine ethyl ester (TMRE)	–
RedoxSensor™ Red CC-1	–	–

13.5 Washing of Spermatozoa

Principle

Washing of spermatozoa is necessitated for estimation of cholesterol content of spermatozoa, in vitro capacitation and acrosome reaction and several other fluorescent assays. For washing of spermatozoa, fresh and frozen-thawed spermatozoa are washed using Percoll density gradient [29] to remove egg yolk particles, dead cells, and debris. However, investigators must bear in mind that not all experimental protocols require washing of spermatozoa.

Materials

Percoll, non-capacitating medium (NCM), and cryo-centrifuge

Percoll 40% and 80%

40/80 mL Percoll
Dilute to 100 mL with NCM

Procedure

(a) Take 1 mL of 80% Percoll in a disposable 15 mL centrifuge tube.
(b) Carefully layer 1 mL of 40% Percoll in test tube containing 80% Percoll.
(c) Now gently layer one mL fresh or thawed semen on top of the two-step Percoll column.
(d) Centrifuge this test tube at 400 g for 30 min.
(e) Throw away supernatant and resuspend the remaining pellet in NCM.
(f) Repeat the first step for washing.
(g) Resuspend the final pellet again in 2 mL NCM.
(h) From this suspension make aliquot of 1 mL containing 100 million spermatozoa in cryovials.
(i) Store cryovials at −20 °C till used further.

13.6 Protocols for Mitochondrial Staining

13.6.1 Rhodamine 123

Principle

Rhodamine 123 was the first dye used to selectively stain functional mitochondria. Rhodamine 123 accumulates within the sperm mid-piece depending upon a transmembrane electrical potential [30]. R123 being a potentiometric membrane dye (builds up as a result of an active electron carrier chain because of oxidative phosphorylation) only fluoresces green when proton gradient over IMM is built up. The flowback of the protons through the ATP synthase to the matrix of the mitochondria leads to ATP production. Collapse of the proton gradient, indicative of the unfunctional mitochondria, results in non-staining of the cell. Thus, unstained sperm is indicative of the absence of functional mitochondria. In contrast, positive-stained cells are aerobically functional [10]. It is quite remarkable to note that an individual sperm either has a fully fluorescent mid-piece or is nonfluorescent, suggesting that depolarization is a sequential, orchestrated event happening simultaneously over

mid-piece [31]. While R123 is highly specific for living cells, it does not possess the ability to differentiate between mitochondria of low and high membrane potential [8]. Also, results are sometimes not sufficiently reproducible without using relatively high amounts of mitochondria [32]. In such cases, the fluorescent R123 then accumulates in the mitochondrial membranes with quenching effect. For the above reasons, R123 for determining mitochondrial membrane potential is seldom used. Another probe of this group, RedoxSensor Red CC-1 stain is sensitive to the superoxide anion [33]. The protocol for evaluating mitochondrial activity in live spermatozoa using fluorescent probes R123 and propidium iodide (PI) is given below.

Materials

Rhodamine 123 (5 mg/mL DMSO, stock solution) and propidium iodide (1 mg/mL DW, stock solution)

Working R123 Solution

30 µL R123 stock solution
120 µL DMSO
Prepare aliquots of 30 µL; wrap and store at −20 °C

Working PI Solution

50 µL stock PI solution
100 µL PBS

Procedure

(a) Take 1 mL semen sample containing 10×10^6 sperm/mL in a 2 mL wrapped cryovial.
(b) Add 3 µL of working R123 solution.
(c) Incubate for 15 min at 37 °C in the dark.
(d) Add 10 µL of working PI solution.
(e) Incubate for 10 min at 37 °C in the dark.
(f) Centrifuge the mixture for 5 min at 500x g.
(g) Discard supernatant and resuspend the sperm pellet in 1 mL PBS.
(h) Place 10 µL of the suspension on microscopic slide and cover it with coverslips.
(i) Examine under epifluorescence microscopy (600x) equipped with digital camera.
(j) Use excitation/barrier filters of 490/515 nm for R123 (blue excitation).
(k) For PI use excitation/barrier filters of 545/590 nm (green excitation).

Determining Optimum Concentration of PI

(a) Take 10×10^6 sperm/mL in a cryovial.
(b) Fix the cells using either with 0.1% saponin (10 min) or 70% ethanol (30 min).
(c) Stain the sperm cells using 0.1–10 µmol/L of PI. A very high concentration of PI will stain both nucleus and cytosol.
(d) Examine under microscope and select concentration giving best images.

Observations

The following fluorescent staining pattern will be visible in the semen smears.

(a) Viable sperm with functional mitochondria: only green fluorescence at the mid-piece
(b) Dead spermatozoa: red fluorescence

Note that Rhodamine 123 shows nonspecific staining of the sperm head (Fig. 13.2).

Points to Ponder

(a) For the above experiment, Olympus CH 30, Tokyo, Japan, can be used.
(b) DMSO solutions can be used for several months. Store working solutions prepared in DMSO under −20 °C.
(c) The working solutions prepared using PBS are unstable; hence, discard after each use.
(d) PI is highly mutagenic. Wear gloves, safety goggles, and mask when handling PI.
(e) Wash the skin immediately copiously once it comes in contact with PI.
(f) In case of contrasting fluorescence from background, wash the pellet once. The background fluorescence is due to hydrolyzed dye remaining in the media.
(g) Always pipette gently to avoid hurting the cells.
(h) Determine the optimum reagent concentration and staining time for best fluorescent images.

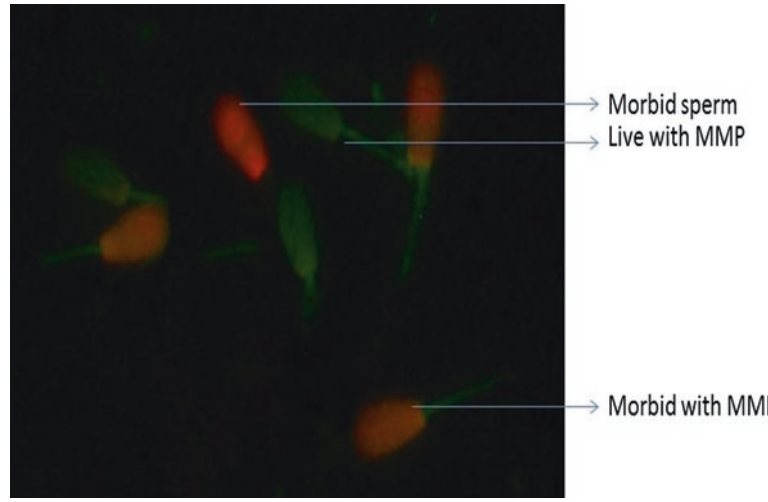

Fig. 13.2 Microphotograph shows spermatozoa stained with Rhodamine 123 and propidium iodide (PI) stains. Green fluorescence of mid-piece shows sperm cells with mitochondrial membrane potential (MMP), dead sperms stained orange/red. Sometimes live sperm heads also take up green fluorescence

13.6.2 JC-1

Principle

JC-1 and JC-9 are two members of the cyanine family. They are dual-emission potential-sensitive (polychromatic) probes that can be used to measure changes in the MMP. The disappearance of the MMP is indicative of early stages of apoptosis. Cossarizza and others [22] used the lipophilic, cationic compound, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) to discriminate mitochondria with low or high membrane potential. Staining with JC-1, a slow dye, leads to formation of multimers known as JC-1 aggregates [34] after accumulation in mitochondria with high MMP or monomers (M-band) in mitochondria with low MMP. In addition to J-aggregates, JC-1 can form monomers (M-band) in mitochondria with low membrane potential. In apoptosis or other events that dissipate the MMP, accumulation of the JC-1 dye in the mitochondria is prevented, and thus, the dye is dispersed throughout the entire cell leading to a shift from red (JC-1 aggregates, multimers) to green fluorescence (JC-1 monomers, apoptotic cells). Thus, JC-1 has the distinct advantage of differentiating cells of high and low mitochondrial membrane potential. The membrane-permeant JC-1 dye is widely used to measure mitochondrial health in apoptotic studies by detection of fluorescence ratio. This permits investigators to make comparative measurement of MMP and thus determine the number of mitochondria within a given cell population that responds to an applied stimulus.

The fluorescence of the cells stained with JC-1 can either be observed by the fluorescence microscopy or measured by the fluorimetric/flow cytometric analysis. In flow cytometry, drawing of two-dimensional green versus red fluorescence (ratio of green to red fluorescence) permits comparative measurements of MMP between cell population. The number of cells with a low MMP could be measured on a biparametric histogram with green vs. red fluorescence or using a red-to-green ratio as a derived parameter [22].

Materials

DMSO, JC-1, and stock staining buffer (HEPES/BSA)

Preparation of Stock Solution (JC-1, 200x, 1mg/mL)

(a) Add 200 μL of DMSO to vial containing 1 mg JC-1.
(b) Close the vial firmly and vertex it.
(c) Leave the solution for 15 min to ensure JC-1 is completely dissolved.
(d) Add DMSO to make up the volume to 1 mL.
(e) Mix thoroughly; prepare working aliquots and store at −20 °C.

Stock Staining Buffer (HEPES/BSA, 5x)
650 mM NaCl
20 mM KCl
70 mM fructose
50 mM HEPES
5 mM $CaCl_2$
2.5 mM $MgCl_2$
0.5% BSA

Staining Buffer (5x/1x)
To prepare 2 mL of the working staining buffer (1x), take 400 μL of the 5x buffer, and make up the volume to 2 mL.

Working Staining Solution (1 mL)

Procedure

(a) Take 5 μL of the stock solution and 800 μL of ultrapure water in 2 mL tube.
(b) Close the tube and mix thoroughly by inversion.
(c) Incubate the tube for 2 min to completely dissolve JC-1.
(d) Add 200 μL of staining buffer (5x) to the tube and mix by inversion.
(e) Procedure described below is for suspension containing 1×10^6 spermatozoa/mL.
(f) Fresh/frozen-thawed semen samples need to be washed before subjecting them to staining.
(g) Mix 1 mL each of the working staining solution and sperm suspension.
(h) Incubate for 20 min at 37 °C in a humidified atmosphere containing 5% CO^2.
(i) Take 2 mL of already prepared working staining buffer and place it on crushed ice.
(j) Centrifuge the sperm suspension (600 x g, 4 min at 5 °C).
(k) Aspirate the suspension and place the tube containing sperm pellet on crushed ice.
(l) Wash the cell pellet with 1 mL of ice-cold working staining buffer.
(m) Resuspend the cells in 1 mL of ice-cold working staining buffer.
(n) Now sperm cells are ready to be evaluated by either fluorescence microscopy or fluorimetric or flow cytometry assay.

13.6.2.1 Fluorescence Determination

13.6.2.1.1 Fluorescence Microscopy

Fluorescence microscopy requires the use of various filters to evaluate different color patterns. The following filters are recommended with JC-1 staining: *in* live cells (JC-1 aggregates, bright red), standard broad-pass filters used for PI can be used; and in apoptotic (JC-1 monomers, green), standard broad-pass filters used for FITC can be used. Dual band-pass filters designed to detect two fluorescent probes simultaneously (e.g., FITC/Cy3) can also be used. Fluorescent microscopes such as Olympus BX 51 and 100x objective using a simultaneous combination of excitation and emission filters at 488/650 nm can be used (Fig. 13.3).

13.6.2.1.2 Fluorimetry

For JC-1 multimers (JC-1 aggregates), set the fluorimeter at 525/590 (ex/em) nm wavelength, and determine the orange fluorescence (first run). For JC-1 monomers, set the fluorimeter at 490/530 (ex/em, optimum is 525 ex) nm wavelength, and determine the green fluorescence (second run). This is followed by blank correction (BC, against TALP containing 2 μM JC-1) of total fluorescence of each well (TF). The means of the TF and BC fluorescence, termed as relative fluorescence units (RFUs), for the two wells is used as the treatment value.

13.6.2.1.3 Flow Cytometry

Use FL 1 and FL 2 channels for detection of JC-1 monomers and aggregates, respectively. Thus, healthy non-apoptotic cells will be detected in both FL1 and FL2 channels (FL1bright, FL2bright), and cells with altered mitochondrial function due to apoptosis or other cellular processes will remain bright in the FL1 channel, but will have reduced FL2 intensity (FL1bright, FL2dim).

Note

(a) The valinomycin contained in the chemicals supplied by the Sigma-Aldrich (USA) permeabilizes (for K+ ions) the mitochondrial

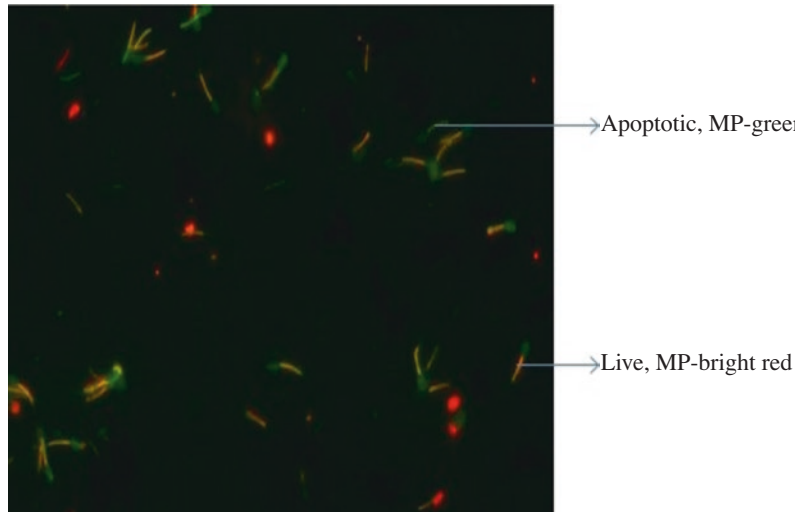

Fig. 13.3 Staining of bovine spermatozoa with JC-1 (40×). Microphotograph showing mid-piece (MP) of bovine spermatozoa stained with JC-1 stain: In live cells with high mitochondrial membrane potential (MMP), bright red fluorescence is observed, whereas in apoptotic spermatozoa mid-piece appears green to light orange in color due to JC-1 monomers

membrane, thereby dissipating the MMP. Thus, it can be used as control that prevents JC-1 aggregation.

(b) For a valinomycin control (mitochondrial gradient dissipation), add 1μL of the valinomycin to the staining solution and mix well.

(c) Ready to use staining buffer (Catalogue No. J3645, Sigma-Aldrich, Bengaluru, India) can also be used.

Observations

JC-1 is a green fluorescent (λex 530 nm) monomer and an orange/red fluorescent (λem 590 nm) at low and high, respectively, MMP in cells. The mitochondrial size, shape, or density do not influence the ratio of red-to-green fluorescence (reversible) of JC-1 which is dependent only on MMP. The potential-sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates.

Points to Ponder

(a) While making working aliquots from the stock solution for any given procedure, quantity should be sufficient for staining all the samples (including the control) to avoid variation.

(b) Since the procedure for staining the sperm cells requires staining buffer of 5 and 1x, do not dissolve entire quantity of stock (5x) staining buffer at once.

(c) The sperm samples should always be kept on crushed ice after staining with JC-1.

(d) No longer than 30 min should elapse between final stage of staining and evaluation.

(e) While evaluating JC-1-stained cells, it is advisable to take image of sperm cells stained red first which fades faster than green fluorescence.

13.6.3 Triple Staining Protocol

The triple staining procedure [35] to evaluate mitochondrial activity as well as intactness of the plasma and acrosome membrane of spermatozoa using combination of JC-1, propidium iodide (PI), and FITC-PSA (fluorescein isothiocyanate-pisum sativum agglutinin) is described below.

Materials

Tyrode's albumin lactate pyruvate (TALP), propidium iodide (0.5mg/mL in PBS), JC-1 (153 μM in DMSO), and FITC-PSA (100 μg/mL in PBS)

Procedure

(a) Dilute fresh semen sample in pre-warmed (37 °C) TALP to a final concentration of 25×10^6 sperm/mL.

(b) Transfer 150 μL of semen sample to a pre-warmed silver foil wrapped microtube.

(c) Add 3 μL of PI, 2 μL of JC-1, and 50 μL of FITC-PSA to the above sample.
(d) Incubate the mixture at 37 °C for 8 min.
(e) Place 7 μL of the stained sample on a cleaned glass slide and put coverslip.
(f) Evaluate immediately under differential interference contrast microscopy.
(g) Count at least 200 cells per slide (in duplicate) and classify sperms as given below.

Observation

Fluorescence pattern exhibited by spermatozoa after staining with triple stain is described below. An investigator is well advised to keep in mind that certain fluorescence pattern might totally be absent from his experimental observations. This might be due to overlapping fluorescence color palette. Moreover, it is sometimes difficult to differentiate red fluorescence emitted from PI- or JC-1-stained spermatozoa with high MMP (Fig. 13.4). The appearance of colorless head with colored tails indicates live cells [36] (Table 13.4).

Points to Ponder

Microscope – Nikon Eclipse 80i: magnification, 1000x with a triple filter (D/F/R, C58420, Nikon) with UV-2E/C (excitation at 340–380 nm and

Table 13.4 Fluorescence pattern exhibited by sperm cells with varying physiological status

Sperm attributes	Fluorescence pattern	Inference
Intact PM, intact AM, high MP	G-Acr + BO-MP	Live, AI with active M
Intact PM, intact AM, low MP	G-Acr + G-MP	Live, AI with inactive M
Intact PM, damaged AM, high MP	LG-Acr + BO-MP	Apoptotic with active M
Intact PM, damaged AM, low MP	LG-Acr + G-MP	Apoptotic with inactive M
Damaged PM, intact AM, high MP	R-N + G-Acr + BO-MP	Morbid, AI with activeM
Damaged PM, intact AM, low MP	R-N + G-Acr + G-MP	Morbid, AI with inactiveM
Damaged PM, damaged AM, high MP	R-N + LG-Acr + BO-MP	Morbid, AR with activeM
Damaged PM, damaged AM, low MP	R-N + LG-Acr + G-MP	Morbid, AI with inactive M

Acr acrosome, *AM* acrosome membrane, *AR* acrosome reacted, *AI* acrosome intact, *BO* bright orange red, *G* green, *LG* light green, *M* mitochondria, *MP* mitochondrial membrane potential, *MP* mid-piece, *N* nucleus, *R* red, *PM* plasma membrane

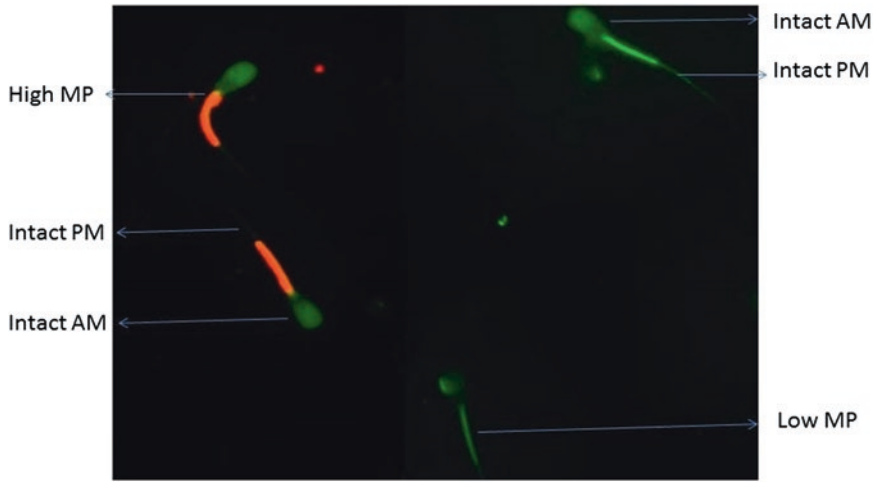

Fig. 13.4 Staining of bovine spermatozoa with triple stain (JC-1+PI+FITC, 40×). Microphotograph showing mid-piece of bovine spermatozoa stained with JC-1 stain: In live cells with high mitochondrial potential (high MP), bright red fluorescence is observed, whereas in apoptotic spermatozoa mid-piece appears green to light orange in color due to JC-1 monomers (low MP). Lectin bound with fluorescein (FITC) has stained acrosome as green (intact AM), whereas intact plasma membrane (PM) is also stained green

emission at 435–485 nm); B-2E/C (excitation at 465–495 nm and emission at 515–555 nm); and G-2E/C (excitation at 540–525 nm and emission at 605–655 nm) filters.

13.6.4 MitoTracker® Probes

Principle

Although conventional fluorescent stains (e.g., tetramethylrosamine and Rhodamine 123) are easily sequestered by functioning mitochondria, these stains wash out of spermatozoa quickly once the MMP is lost. This drawback limits the application of such stains in procedures requiring cellular treatment with aldehyde fixative or agents which affect MMP. This limitation is overcome by application of MitoTracker probes – patented mitochondrion-specific dyes that accumulate in high MMP even when cells are fixed [37] (MitoTracker® Mitochondrion-Selective Probes, Molecular Probes, Invitrogen, Paisley, UK).

The cell-permeant MitoTracker® probes have a mildly thiol-reactive chloromethyl moiety for labeling mitochondria. This group appears to keep the dye associated with the mitochondrial cytosols after fixation [28]. MitoTracker® probes are derivative of either tetramethylrosamine or carbocyanine compound: MitoTracker® probes such as rosamine-based dyes, viz., MitoTracker® Orange CMTMRos (derivative of tetramethylrosamine) and MitoTracker® Red CMXRos (derivative of X-rosamine); reduced probes, viz., MitoTracker® Orange CM-H2TMRos and MitoTracker® Red CM-H2XRos (derivatives of dihydrotetramethylrosamine and dihydro-X-rosamine, respectively); and MitoTracker® Deep Red, MitoTracker® Red FM, and MitoTracker® Green FM (carbocyanine-based MitoTracker® probes); the stained sample retains the fluorescent pattern characteristic of viable cells. This reduced probes do not show fluorescence until after they enter live spermatozoa, whereupon they are oxidized to form the respective fluorescent mitochondrion-selective probe followed by their sequestration in the mitochondria. Moreover, MitoTracker® Green FM and MitoTracker® Red FM are useful to stain live cells as well but are not retained after cell fixation. Several MitoTracker® probes with red fluorescence (well resolved from green fluorescence of other probes) such as MitoTracker® Red CMXRos, MitoTracker® Deep Red FM, and MitoTracker® Red FM are suitable for multicolor labeling experiments.

Materials

DMSO (anhydrous), formaldehyde, and MitoTracker® Red CMXRos

Stock Solutions (1 mM)

50 µg MitoTracker®® Red CMXRos
Dilute to 1 mL of DMSO

Working Solutions (100 nM) (for Cells Requiring Fixing and Permeabilization)

Procedure

(a) Take semen sample containing 10^6 cells.
(b) Wash the sample as described before.
(c) Carefully aspirate and discard the supernatant.
(d) Resuspend the cells gently in 1 mL of working probe solution (37 °C) by vortexing.
(e) Incubate for 30 min at (37 °C).
(f) After staining, add 1 mL of PBS (37 °C) to the suspension; re-pellet the spermatozoa by centrifugation (500 x g for 5 min).
(g) Resuspend the cells gently in 1 mL of PBS containing 2% formaldehyde.
(h) Incubate the suspension at 37 °C for 15 min.
(i) Permeabilize the cells by incubating in ice-cold acetone for 5 min.
(j) Rinse the cells two times in PBS.
(k) Examine under fluorescence microscope as described before.

Observations

Living sperm cells will show red fluorescence. However, it is advisable to combine this probe with other viability probes used for viability studies, e.g., SYBR-14 to differentiate between apoptotic and viable cell number.

Points to Ponder

(a) The rosamine MitoTracker® probes once reduced turn sensitive to oxidation in solution. Therefore, storage under argon or nitrogen at ≤20 °C (may be in liquid nitrogen) protected from light is a must.
(b) Reduced MitoTracker® probes require comparatively higher concentration than other MitoTracker®® probes to stain sperm cells.
(c) Do not store working solutions of dihydro derivatives; use immediately after preparation.
(d) MitoTracker® green dyes stain other cellular structures at higher concentrations.
(e) Always use warm (37 °C) solutions for staining purposes.
(f) Permeabilization of sperm cells improves fluorescence of the cells.

13.6.5 MTT Reduction Assay

Principle

This assay exploits ability of living and metabolically active spermatozoa to reduce yellow water-soluble salt tetrazolium (3[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) or MTT, to formazan, a water-insoluble purple compound. This is achieved by the reductive cleavage of its tetrazolium ring in the active mitochondria in the succinate dehydrogenase system [38]. The procedure involves determining the amount of formazan spectrophotometrically and serves to indicate the number of functional mitochondria and thus live cells, in a sample [39]. This is comparable to resazurin redox dye and methylene blue reduction assay described elsewhere in this book. The procedure given below is adapted from the protocol described by Mosmann [40].

Materials

Dilution buffer (HEPES buffer with 1% BSA) and MTT stock solution (5 mg MTT/mL of PBS

Procedure

(a) Take a semen sample containing ≥70% of viable spermatozoa.
(b) Determine sperm cell concentration using hemocytometer.
(c) Dilute the test semen sample with dilution buffer at 1:1 ratio.
(d) Divide the sample in 10 tubes containing 200–2000 x 10^6 cells serially.
(e) Make up the volume in each tube to 2 mL using dilution buffer.
(f) Mark them S1–S10.
(g) Take 2 mL of dilution buffer in another tube and mark it B (blank).
(h) Set the spectrophotometer reading (550 nm) to zero using blank (B).
(i) Add 200 μL of MTT stock solution to tubes marked S1–S10.
(j) Take optical density (OD) of the samples S1–S10 immediately.
(k) Incubate the samples at 37 °C for 1 h and take OD.
(l) The reduction rate of MTT for individual sample is arrived at by deducing the difference between the first and second reading (OD).
(m) Plot a standard curve using sperm concentration vs. OD reading (X and Y axes, resp.).
(n) Dilute the test semen sample (200 μL) with dilution buffer (1.8 mL); mark it T.
(o) Add 200 μL of MTT stock solution; mix well and take OD.
(p) Incubate the test sample at 37 °C for 1 h and take OD.
(q) Calculate the difference between two readings, and using standard curve, calculate mitochondrial function/viability of the test sample.

Observations

The MTT reduction rate (OD) at 550 nm after 1 h incubation at 37 °C ranges from zero to one.

Points to Ponder

Absolute cleanliness of the test tubes used is a must for accurate results.

13.7 Background Information

Mitochondria (mito, filaments; chondria, grains) are important cellular organelle involved in the survival of eukaryotic cells including sperm cells. They perform essential cellular functions to maintain homeostasis within and in the metabolism of lipids and proteins [41]. Like Janus, mitochondria show two aspects of cellular survival; i.e., it is associated with continuation of viability and vitality in addition to playing a crucial central role in the regulation of apoptosis [4], standing like a keeper of the passage between death and life.

It is apparent that evaluating mitochondrial function is crucial to determine sperm health. Over passage of time, several techniques and probes have been developed to evaluate mitochondrial function without destroying the cell (timeline, Figure 10.7). Various mitochondrion-specific cationic dyes tend to accumulate within the spermatozoa and more particularly localize in the mitochondrial matrix, induced by the MMP (refers to potential difference between the intra- and extracellular aqueous phases or resting potential).

Property of some of the dyes to form covalent bonds with mitochondrial proteins enhances their compatibility with fixation step in cellular staining. This is important in instances where samples are to be examined after some lag. In such experiments, formaldehyde followed by acetone is most suited for subsequent permeabilization [42]. The latter agent permits intracellular labeling using antibodies and also improves intracellular retention of the probe. However, these procedures (fixation or permeabilization) cannot be applied to staining with MitoTracker® Green FM (Table 13.5).

While using a fluorescent probe, an investigator is advised to use threshold level (unique for all dyes) of that particular probe; any concentration above this results in fluorescence quenching.

Table 13.5 Chronology of emergence of fluorescence probes for assessing mitochondrial functionality

2002	MitoSOX	Batandier et al. (2002)	[43]
2001	Carboxy SNARF-1	Takahashi et al. (2001)	[44]
2000	MitoFlour	Keij et al. (2000)	[45]
	RedoxSensor Red CC-1	Chen and Gee (2000)	[33]
1996	MitoTracker®	Poot et al. (1996)	[46]
1991	JC-1/JC-9	Reers et al. (1991)	[47]
		Smiley et al. (1991)	[9]
1990	Styryl dyes	Bereiter-Hahn (1990)	[48]
1988	TMRM/TMRE	Ehrenberg et al. (1988)	[23]
1983	NAO	Septinus et al. (1983)	[20]
1981	Carbocyanines	Johnson et al. (1981)	[49]
1980	Rhodamine 123	Johnson et al. (1980)	[12]
1900	Janus Green B	Michaelis (1900)	[50]

Adapted from Cottet-Rousselle et al. [14]

Fluorescent quenching results in signal from matrix becoming largely independent of excess accumulation of cationic probes. In cells loaded with higher concentrations than this threshold, fluorescence quenching in the cells results in artifacts in the MMP signal. In view of the above information, a researcher must consider the effect of the fluorescent dye distribution along the spermatozoa plasma membrane. Following rapid mitochondrial depolarization and subsequent redistribution of the fluorescent probe from accumulated (quenched) matrix, a transient high fluorescence in the cytoplasm will be produced. This cytoplasmic quenching will eventually decay as the probe redistributes across sperm plasma membrane [51]. This property of the cationic fluorescent probes mandates that a researcher must use a third to fourth of the recommended initial loading concentration in the new buffer or medium for even distribution [52].

Depending on the type of the probe and concentration, application of mitochondrion-specific fluorescent probes induces inhibition of sperm mitochondrial respiration (none with TMRM,

Table 13.6 Troubleshooting

Problem	Probable cause	Solution
Cells improperly stained	Damaged dye due to prolonged storage	Check expiry, and discard
	The working solution not properly prepared or is exposed to light	Prepare fresh working solution
	The concentration of working solution too low	Use proper solvent to prepare recommended concentration
Efflux of dye from cells	A physiological function of cell	Examine stained cells quickly
	Insufficient reagent	Probenecid, a transport inhibitor, may be used
Probe not dissolved properly	Dye may be sticking at the bottom	Use a vortex mixer or ultrasonic bath to dissolve the dye
	Damaged dye	Check expiry, and discard
	Wrong solvent	Use recommended solvent
High fluorescent background	Insufficient washing	Repeat washing with PBS
	Too much dye	Prepare recommended concentration

intermediate with R123 and TMRE, and strongest inhibition with DiOC6(3)). Because of toxic inhibitory action of the fluorescent probes, it is advisable to use low amounts at recommended concentration only [53].

On the other hand, it has been observed that some mitochondria, but not all within same ejaculate, loss their MMP during apoptosis or under influence of depolarizing agents. This suggests that reduced MMP is not an absolute indicator of cell death. Cellular quiescence or stress causes inhibition of respiration due to temporary mitochondrial membrane permeabilization followed by apoptosis. Detection of apoptotic cells in a given sample must be confirmed by other specific techniques, e.g., detection of activated caspases (Table 13.6).

Literatures Cited

1. Hecht NB, Liem H, Kleene KC, Distel RJ, Ho SM (1984) Maternal inheritance of the mouse mitochondrial genome is not mediated by a loss or gross alteration of the paternal mitochondrial DNA or by methylation of the oocyte mitochondrial DNA. Dev Biol 102:452–461
2. Olson GE, Winfrey VP (1992) Structural organization of surface domains of sperm mitochondria. Mol Reprod Dev 33:89–98
3. Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF (2004) Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. Proc Natl Acad Sci U S A 101:16501–16506
4. Marin S, Chiang K, Bassilian S, Lee WN, Boros LG, Fernandez-Novell JM (2003) Metabolic strategy of boar spermatozoa revealed by a metabolomic characterization. FEBS Lett 554:342–346
5. Silva PF, Gadella BM (2006) Detection of damage in mammalian sperm cells. Theriogenology 65(5):958–978
6. Gadella BM, Harrison RA (2002) Capacitation induces cyclic adenosine 30, 50-monophosphate-dependent, but apoptosis-unrelated, exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. Biol Reprod 67:340–350
7. Terrell KA, Wildt DE, Anthony NM, Bavister BD, Leibo SP, Penfold LM (2012) Different patterns of metabolic cryo-damage in domestic cat (Felis catus) and cheetah (Acinonyx jubatus) spermatozoa. Cryobiology 64(2):110–117
8. Gravance CG, Garner DL, Baumber J, Ball BA (2000) Assessment of equine sperm mitochondrial function using JC-1. Theriogenology 53:1691–1703
9. Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, Steele GD, Chen LB (1991) Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate-forming lipophilic cation JC-1. Proc Nat Acad Sci U S A 88:3671–3675
10. Garner DL, Thomas CA, Joerg HW, DeJamette JM, Marshall CE (1997) Assessment of mitochondrial function and viability in cryopreserved bovine sperm. Biol Reprod 57:1401–1406
11. Rottenberg H, Wu S (1998) Quantitative assay by flow cytometry of the mitochondrial membrane potential in intact cells. Biochim Biophys Acta 1404:393–404
12. Johnson LV, Walsh ML, Chen LB (1980) Localization of mitochondria in living cells with rhodamine 123. Proc Natl Acad Sci U S A 77:990–994
13. Fraser L, Zasiadczyk L, Strzezek J (2010) Interactions of egg yolk lipoprotein fraction with boar spermatozoa assessed with a fluorescent membrane probe. Folia Histochem Cytobiol 48(2):292–298
14. Cottet-Rousselle C, Ronot X, Leverve X, Mayol J (2011) Cytometric assessment of mitochondria using fluorescent probes. Cytometry A 79A:405–425

15. Metivier D, Dallaporta B, Zamzami N, Larochette N, Susin SA, Marzo I, Kroemer G (1998) Cytofluorometric detection of mitochondrial alterations in early CD95/Fas/APO-1-triggered apoptosis of Jurkat T lymphoma cells. Comparison of seven mitochondrion-specific fluorochromes. Immunol Lett 61:157–163
16. Petit PX, O'Connor JE, Grunwald D, Brown SC (1990) Analysis of the membrane potential of rat- and mouse-liver mitochondria by flow cytometry and possible applications. Eur J Biochem 194:389–397
17. Gregory J (2002) Molecular Probes. Handbook of fluorescent probes and research products. Molecular Probes Inc, Eugene
18. Hallap T, Nagya S, Jaakmac U, Johannisson A, Rodriguez-Martinez H (2005) Mitochondrial activity of frozen-thawed spermatozoa assessed by MitoTracker® Deep Red 633. Theriogenology 63:2311–2322
19. Septinus M, Berthold T, Naujok A, Zimmermann HW (1985) Hydrophobic acridine dyes for fluorescent staining of mitochondria in living cells. 3. Specific accumulation of the fluorescent dye NAO on the mitochondrial membranes in HeLa cells by hydrophobic interactions. Depression of respiratory activity, changes in the ultrastructure of mitochondria due to NAO. Increase of fluorescence in vital stained mitochondria in situ by irradiation. Histochemistry 82:51–66
20. Septinus M, Seiffert W, Zimmermann HW (1983) Hydrophobic acridine dyes for fluorescence staining of mitochondria in living cells. 1. Thermodynamic and spectroscopic properties of 10-n-alkylacridine orange chlorides. Histochemistry 79:443–456
21. Maftah A, Petit JM, Julien R (1990) Specific interaction of the new fluorescent dye lo-N-nonyl acridine orange with inner mitochondrial membrane A lipid-mediated inhibition of oxidative phosphorylation. FEBS Lett 260(2):236–240
22. Cossarizza A, Baccarani-Contri M, Kalashnikova G, Franceschi C (1993) A new method for the flow cytometric analysis of mitochondrial membrane potential using the J-aggregate forming lipophilic cation 5,5′,6,6′-tetrachloro-l,l′,3,3′-tetraethylbenzimidazolyl carbocyanine iodide (JC-1). Biochem Biophys Res Commun 197:40–45
23. Ehrenberg B, Montana V, Wei MD, Wuskell JP, Loew LM (1988) Membrane potential can be determined in individual cells from the nernstian distribution of cationic dyes. Biophys J 53:785–794
24. Scaduto RC Jr, Grotyohann LW (1999) Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J 76:469–477
25. Farah OI, Cuiling L, Jiaojiao W, Huiping Z (2013) Use of fluorescent dyes for readily recognizing sperm damage. J Reprod Infertil 14(3):120–125
26. Naser-Esfahani MH, Aboutorabi R, Esfandiari E, Mardani M (2002) Sperm MTT viability assay: a new method for evaluation of human sperm viability. J Assoc Reprod Genet 19:477–482
27. Aziz DM (2006) Assessment of bovine sperm viability by MTT reduction assay. Anim Reprod Sci 92:1–8
28. Piccoli C, Boffoli D, Capitanio N (2004). Comparative analysis of mitochondria selective dyes in different cell types detected by Confocal Laser Scanning Microscopy: methods and applications. Current Issues on Multidisciplinary Microscopy Research and Education. FORMATEX 2004: 130–139. Internet download dated 20 July 2016
29. Ward CR, Storey BT (1984) Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. Dev Biol 104:287–296
30. Evenson DP, Darzynkiewicz Z, Malamed MR (1982) Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to sperm motility. J Histochem Cytochem 30:279–280
31. Mukai C, Okuno M (2004) Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. Biol Reprod 71:540–547
32. Troiano L, Granata AR, Cossarizza A, Kalashnikova G, Bianchi R, Pini G, Tropea F, Carani C, Franceschi C (1998) Mitochondrial membrane potential and DNA stainability in human sperm cells: a flow cytometry analysis with implications for male infertility. Exp Cell Res 241:384–393
33. Chen CS, Gee KR (2000) Redox-dependent trafficking of 2,3,4,5,6-Pentafluorodihydro tetramethylrosamine, a novel fluorogenic indicator of cellular oxidative activity. Free Radic Biol Med 28:1266–1278
34. Jelley EE (1937) Molecular, nematic and crystal states of 1: 1 '-diethyl-cp-cyanine chloride. Nature 139:631–632
35. Celeghini ECC, Nascimento J, Andrade AFC, Raphael CF, Souza LWO, Arruda RP (2005) Use of CMXRos and JC-1 on mitochondrial function evaluation, associated to fluorescent probes to plasmatic and acrosomal membranes evaluation in bovine spermatozoa. Acta Sci Vet 33:321
36. Lange-Consiglio A, Meucci A, Cremonesi F (2013) Fluorescent multiple staining and CASA system to assess boar sperm viability and membranes integrity in short and long-term extenders. Open Vet J 3(1):21–35
37. MitoTracker® Mitochondrion-Selective Probes, E-booklet, Internet 19 July 2016. Molecular Probes, Invitrogen, Paisley
38. Slater TF, Swyer B, Sträuli U (1963) Studies on succinate-tetrazolium reductase systems. III Points of coupling of four different tetrazolium salts. Biochim Biophys Acta 77:383–393
39. Denizot F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. J Immunol Methods 89:271–277
40. Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63

41. Solaini G, Sgarbi G, Lenaz G, Baracca A (2007) Evaluating mitochondrial membrane potential in cells. Biosci Rep 27:11–21
42. Kroemer G, Galluzzi L, Brenner C (2007) Mitochondrial membrane permeabilization in cell death. Physiol Rev 87:99–163
43. Batandier C, Fontaine E, Keriel C, Leverve XM (2002) Determination of mitochondrial reactive oxygen species: methodological aspects. J Cell Mol Med 6:175–187
44. Takahashi A, Zhang Y, Centonze E, Herman B (2001) Measurement of mitochondrial pH in situ. Biotechniques 30:804–808. 810,812 passim
45. Keij JF, Bell-Prince C, Steinkamp JA (2000) Staining of mitochondrial membranes with 10-nonyl acridine orange, MitoFluor Green, and MitoTracker® Green is affected by mitochondrial membrane potential altering drugs. Cytometry 39:203–210
46. Poot M, Zhang YZ, Kramer JA, Wells KS, Jones LJ, Hanzel DK, Lugade AG, Singer VL, Haugland RP (1996) Analysis of mitochondrial morphology and function with novel fixable fluorescent stains. J Histochem Cytochem 44:1363–1372
47. Reers M, Smith TW, Chen LB (1991) J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 30:4480–4486
48. Bereiter-Hahn J (1990) Behavior of mitochondria in the living cell. Int Rev Cytol 122:1–63
49. Johnson LV, Walsh ML, Bockus BJ, Chen LB (1981) Monitoring of relative mitochondrial membrane potential in living cells by fluorescence microscopy. J Cell Biol 88:526–535
50. Michaelis L (1900) Die vitale Farbung, eine Darstellungsmethode d. Zell-granula. Arch f miki Anat 55:558–575
51. Nicholls DG, Ward MW (2000) Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts. Trends Neurosci 23:166–174
52. Lemasters JJ, Ramshesh VK (2007) Imaging of mitochondrial polarization and depolarization with cationic fluorophores. Methods Cell Biol 80:283–295
53. Gravance CG, Garner DL, Miller G, Berger T (2001) Fluorescent probes and flow cytometry to assess rat sperm integrity and mitochondrial function. Reprod Toxicol 15(1):5–10

Key References

54. Cottet-Rousselle and co-workers, 2011. See above [14] In-depth discussion and excellent background information related to application of fluorescent probes for detection of mitochondrial activity in a cell population
55. Piccoti et al. (2004). See above [28] Provides comparative merits/demerits of some of the fluorescent dyes used in the evaluation of mitochondrial activity
56. Celeghini and others (2005) [35] and Lange-Consiglio and co-workers (2013). See above [36]. Contains a detailed alternate procedure for triple staining of sperm cells for simultaneous evaluation of plasma, acrosome integrity and mitochondrial function without intervening process of washing
57. Aziz (2006). See above [27] Describes MTT reduction assay to evaluate mitochondrial function