The diagnostic and therapeutic applications of flow cytometry in male infertility

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Abstract

Although in recent years flow cytometry (FCM) has become common place in clinical haemotopathology laboratories, application of this technology in the andrology laboratory remains largely unrealized. Flow cytometry is the semi-automated study of the antigen profile of cells using the Scatchard principle of antigen-antibody binding and fluorochrome-based detection systems. Recent technical advances in lasers, monoclonal antibodies, fluorochromes, and computer-based color compensation algorithms have expanded the usefulness of FCM. This overview presents the background of FCM, discusses various applications, and speculates on the directions of future developments with special relevance to the field of andrology. The availability of high-quality methods should be a prime factor in convincing andrologists and infertility clinics that FCM may have certain advantages over traditional methods and that it does indeed have much to contribute to the andrology laboratory and male infertility clinic.

Key words: andrology, fluorescence, laboratory, sperm.

Introduction

Infertility management is nowadays improved due to the advent of assisted reproductive technologies (ART) and the introduction of a variety of *in vitro* fertilization (IVF) techniques. However, the sperm quality is critical in determining the success of ART. Intracytoplasmic sperm injection (ICSI), which bypasses natural selection, may inject sperm that would fail during the natural course of conception [1]. This raises concerns about the genetic risks that can be involved in ART, especially ICSI [2]. Sperm with altered DNA or other molecular-level damage may be selected for ICSI by chance and used for oocyte injection, resulting in poor ART outcome.

Over the last decade, a number of laboratory tests have been developed to examine sperm function. These include quantitative sperm motility parameters, capacitation, basal and induced acrosome reactions, sperm-zona pellucida interactions, and nuclear and mitochondrial sperm DNA. Only few of them have been adopted into routine clinical use [3]. On the other hand, efforts to improve sperm quality for ICSI and to find an automated objective analysis method with low operator variability continue [4-7]. Flow cytometry (FCM) has developed into a highly reliable and

convenient technology that can serve the andrology lab and infertility practices.

The adaptation of FCM to sperm assessment began initially as a method for measuring DNA content [8, 9]. Flow cytometry can be now applied to semen evaluation of sperm count, viability, acrosomal reaction, mitochondrial membrane integrity, intracellular reactive oxygen species (ROS), calcium flux, capacitation status, membrane fluidity, and chromatin status. New fluorescent stains and techniques that have potential application to the flow cytometric evaluation of spermatozoa are continuously being developed. Flow cytometry applications may also serve as a useful tool in reproductive toxicology (to study the effects from environmental, occupational and therapeutic exposures), in forensic medicine (to identify and sort sperm cells from vaginal wash in alleged rape), gender pre-selection (for the animal industry and in some sex-linked diseases), in clinical andrology (to assess sperm quality, leukocytospermia, malignant cell and/or to predict ART outcomes), and in contraceptive evaluation (to detect the minimal dose that minimizes fertilization potential) [1, 10, 11]. In this review, we discuss the possible diagnostic and therapeutic roles of FCM in the andrology lab and male fertility setting.

Flow cytometry: history and overview

Flow cytometry is a precise, sensitive, accurate, rapid, and dynamic method of multiparameter, single-cell analysis. This technique provides statistical accuracy and reproducibility [12]. Flow cytometry dates back to the early 1970s as it was developed to enhance the microscopic analysis of individual cells and to facilitate sorting of specific cell populations from other cells using fluorescent probes [13-15]. The advent of air-cool lasers and the refinement of rigid optical systems facilitated the emergence of high-speed, bench-top, multiparameter fluorescence-activated cell sorting (FACS) [12, 14, 16]. The growing availability of fluorescent probes has stimulated interest in a variety of methods for addressing many properties of sperm [17-20].

Instrumentation principles

Flow cytometry may be combined with electrical or mechanical FACS. The main components are a fluidics system for hydrodynamically focusing a single cell to the laser excitation site, a proper laser source for single-cell excitation, an optics system for proper scattered light collection by dichroic mirror and filters, and electronic capability for data acquisition and analysis [14, 21-25]. Based on the medium that is used to amplify the light for laser generation, it may be gas (argon, helium laser), solid sate (YAG laser), dye or semiconductor laser.

Cell suspension is directly or indirectly labeled with one or more fluorescent probes. In a flow system, cells are hydrodynamically focused in a single cell line surrounded by the sheath fluid [21, 22, 26, 27]. Each cell, excited by the proper laser, is measured for light scatter signals (physical and granularity cell properties) and emitted fluorescent light. The optics system collects and amplifies the collected light scatter and fluorescent signals by the photomultiplier tubes (PMT). The recorded signals are converted into data files that contain the proper data from the analyzed cells. There are many software programs that can analyze the acquired data files [14, 22].

Fluorochrome probes

Fluorescent-conjugated monoclonal antibodies are the routine probes used in clinical FCM for simple multicolor direct labeling studies. Indirect labeling with a secondary conjugated antibody can also be used. Based on the suitable excitation laser beam, FCM probes can be grouped into: (1) 488 nm blue laser-excited group, including fluorescein isothiocyanate (FITC), phycoerythrin (PE), propidium iodide (PI), 7-amino-actinomycin D (7AAD), peridin-chrolophyl-A-protein (PerCP), dimmers of thiazole orange (TOTO-1), and Alexa Fluor® conjugates; (2) 635 nm red laser-excited group such as allophycocyanin (APC); (3) 405 nm violet laser-excited group, including DNA dyes 4,6-diamidno-2-phenylindole (DAPI) or Hoechst 33342 dye, and calcium flux indole reagent [11, 12, 22, 28, 29].

Specimen preparation/staining

The cell suspension should be homogenous without any debris. Tissue biopsy should be prepared by physical or chemical methods to obtain as many cells as possible from the biopsy. Tissue biopsy may require red blood cell lysis before the staining procedure. Each assay procedure has a different staining protocol [30-33]. Three basic protocols and their appropriate combinations are available to strategically stain the cells for FCM analysis: (1) a primary antibody followed by a fluorochrome conjugated to a second antibody (FL-Ab); (2) a biotinylated primary antibody (B-Ab) followed by a fluorochrome-labeled avidin (FL-Av); (3) fluorochrome conjugated to the primary antibody [34]. Artifactual data may result when broad emission dyes such as PI overcomes the dimly stained (PE-MAb) cells. For better resolution of these cells, narrow spectrum dyes such as 7AAD should be used instead of PI. For combined surface and internal markers analysis, surface marker staining should be done before cell permeabilization and/or fixation for internal staining [34, 35].

Data acquisition and analysis

The physical and chemical properties of the analyzed cells are recorded during acquisition of up to 50,000 data events. Establishment of primary performance by using microbead calibrators and/or standards is recommended for achieving confidence in the tested specimen results as well as quality control purposes [14, 36, 37]. Gating on the desired cell population and the use of suitable graphical presentation are the heart of flow cytometric data. Flow cytometry data presentation may be in single-marker or two-colored histograms. A two-parameter histogram can be presented with dot or contour density plots with the addition of color [38-40]. Monitoring of instrument setup, performance, optimizing staining protocol and reagent/specimen preparation, and standardizing the controls and data interpretation are essential for reliable quality assurance. The use of quantitative FCM is possible by the use of fluorescence-standard curves in units of molecular equivalents of soluble fluorochrome [41-44].

Spermatozoa as a challenging cell for flow cytometry

Accurate detection of a sperm marker depends on the ability of bench-top FCM to precisely orient the spermatozoa at the time of measurement in the flow cell of the analyzer or sorter. The sperm wave generated after exposure to the laser beam shows a characteristic pattern due to the presence of the tail following the head for each spermatozoon. Sperm may be stained by single or multiple markers for accurate FCM detection of sperm attributes. The staining quality for each sperm compartment should be verified by fluorescent microscope [18].

Sperm cells with oval heads tend to be more readily oriented in a sorter using hydrodynamics different from those used for rounded or angular head spermatozoa. Flow cytometry provides information about sperm physical characteristics, such as cell size, shape, and internal granularity, and any component or function of the spermatozoa that can be detected by fluorochrome labeling [11].

Most of the common probes in somatic cells can be applied to sperm FCM analysis. However, some require dose titration and standardization before usage for research or clinical trials in the andrology lab. The most common probes used for FCM sperm analysis include Hoechst 33342, annexin V, SYBR 14, fluorescent-labeled plant lectines, CD 46, CD100, labeled antibodies against ubiquitin, fluorescein isothiocyanate-deoxyuridine-5'-triphosphate (FITC-dUTP), acridine orange, dihydroethiudium bromide (DHE), dichlorofluoracien diacetate (DCFH-DA), rhodamine123, JC-1, YO-PRO-1, and propidium iodide (PI) [18, 45-47].

Male infertility evaluation

Semen flow cytometry analysis for spermatogenetic defects

Ejaculated sperm analysis was studied in normozoospermic, oligozoospermic, and azoospermic semen specimens [48]. Flow cytometry histogram findings from ejaculates showed patterns that correlated with the testicular histology. Some researchers examined ejaculated sperm for conventional parameters using FCM, others examined for new single or multiple markers. Most of the researchers added new markers and correlated them with sperm quality, fertility potential, and ART outcome [1, 11].

Testicular biopsy flow cytometry analysis

Flow cytometric ploidy analysis may provide great help for predicting the success of testicular sperm retrieval verifying the results of wet preparation for sperm or spermatid presence, and diagnosis of testicular tumors and sperm aneuploidy. Cytometry ploidy analysis was recommended before testicular sperm extraction and whenever no sperm are available [48, 49].

Sperm sorting by flow cytometry

Sperm selection by FCM can be based upon specific parameters that differentiate between sperm subpopulations [50, 51]. Sorting parameters may include one or more physical, chemical, or fluorescent markers of the spermatozoa. Flow cytometry sperm sorting may be indicated for research purposes as *in vitro* studies on a specific sperm population e.g, annexin V-negative, TdT-mediated dUTP Nick-End Labeling (TUNEL)-negative, or high mitochondrial membrane potential sperm populations. Another indication for FCM sperm sorting is for clinical use such as sperm sexing/microsorting, based on the DNA content differences between X- or Y-sperm carrying chromosomes [52-54].

Multi-parameter flow cytometry sperm analysis

A mixture of fluorescent-labeled antibodies can be used for explicit identification of sperm subpopulations. Viable spermatozoa with specific intracellular ROS measurement was recently reported (Mahfouz et al. 2008). Examination of viability, acrosomal integrity and mitochondrial membrane potential can be also assessed by proper staining using different fluorochromes [11, 45]. Multi-parameter FCM requires initial settings for properly gating target cells to exclude cell debris. Proper instrument alignment, quality microspheres, and compensation are essential to accurately analyze the data and getting accurate results [55].

Potential flow cytometric andrology assays

Sperm count

The principle of performing sperm count using FCM is based on the mixing the semen specimen with a known volume of fluorescent microbeads of known concentration added as internal standard in each specimen. Results can be calculated accurately by using this equation: Cv = (Cb × Vb)/Vs, where Cv = specimen concentration (unknown), Cb = beads concentration (known), Vb = volume used from microbeads suspension. This equation applies when the total volume of semen/bead mixture was used completely for analysis. A standard curve using known sperm concentrations with a fixed bead number may be used for obtaining FCM-based sperm concentration [56, 57].

Sperm surface markers

Some sperm surface targets may be used to assess sperm quality. The progesterone receptor (PR) located on the sperm surface is one of such targets. Gadkar et al. [58] found fewer PR-positive sperm by FCM in men with oligozoospermia, asthenozoospermia, oligoasthenozoospermia, and teratozoospermia compared with normozoospermic men. Recently, it has been reported that defective spermatozoa become surface-ubiquitinated during epididymal maturation, adding new markers for sperm quality assessment. Sutovsky et al. reported differential ubiquitination of spermatozoa during epididymal maturation and that this ubiquitination may reflect changes in sperm number and semen quality [59].

Changes in membrane fluidity detected by merocyanine 540 (M540) are believed to precede the calcium influx detected by chlortetracycline (CTC), making M540 a better method for evaluating the early events of capacitation [60]. Incorporation techniques using labeled phospholipid analogs and binding proteins also can be used to investigate the phospholipid changes occurring in the plasma membrane of spermatozoa. Annexin V is a calcium-dependent phospholipid-binding protein that has a high affinity for phosphatidylserine (PS) and when conjugated to a fluorochrome, such as FITC, permits the recognition of cells with exposed PS. Similarly, labeled Ro-09-0198 can be used to detect surface exposure of phosphatidylethanolamine (PE). Gadella and Harrison used FITC-annexin V and labeled Ro-09-0198 to demonstrate that bicarbonate induces the exposure of PE and PS on the surface of boar spermatozoa [61].

Sperm viability/plasma membrane integrity testing

Sperm viability can be tested by using fluorescent staining in two ways: fluorochromes used to indicate viable cells and those used to indicate non-viable cells. Flow cytometry can determine the percentages of each subpopulation. Viable sperm can be distinguished by staining with PI, alone or in association with other dyes such as SYBR14, YO-PRO-1 or annexin V [62]. Combinations may differentiate the sperm cells into three or four subpopulations: viable, apoptotic (early and late), and necrotic. Viability of cryopreserved sperm using PI was the single laboratory assay that correlated with stallion fertility (r=0.68) [63]. Consistently, viability assayed by H33258 negatively correlated with fertility (r=–0.57)[64]. Viability stains can be used in combination with stains for assessing other sperm parameters by FCM, such as acrosomal integrity or mitochondrial function [11].

Sperm motility

Reports showed mitochondrial membrane potential markers may be a good indicator for the sperm motility as demonstrated by using rhodamine 123, nonyl-acridine orange and JC-1 [1, 45].

Leukocytospermia

Presence of more than 1 million leukocytes/ml semen indicates leukocytospermia and signifies urogential tract infection. Detection and count of the leukocytes in semen can be done by FCM using monoclonal antibodies against CD45 and CD15 [45, 65-67].

Intracellular calcium level

Sperm function has been assessed using specific calcium dyes in conjunction with FCM [68]. The increase in intracellular free calcium believed to be associated with capacitation can also be detected using molecules that penetrate aqueous compartments and change their spectra when bound to calcium, for example, fluo-3-acetomethoxy (AM) ester, fura red-AM ester, quin-2 AM and indo-1 AM. Using fluo-3 detected by FCM, boar spermatozoa cooled slowly to 5°C and re-warmed to 39°C exhibited increases in intracellular calcium that were similar to those of capacitated spermatozoa [69, 70].

Sperm mitochondrial membrane integrity

Rhodamine 123 (R123) and MitoTracker® (MITO) have been used to evaluate the mitochondrial membrane integrity of spermatozoa. All functioning mitochondria stain green with R123 and MITO dyes. Mitochondrial function assessed by R123 or MITO probes was found to correlate well between both methods used and also with SYBR-14 assessment of viability and microscopic estimation of motility. MITO-labeled mouse spermatozoa were placed in the female reproductive tract by artificial insemination to trace the distribution of the mitochondria in the developing embryo [71, 72].

The mitochondrial stain 5, 50, 6, 60-tetrachloro-1,10,3,30-tetraethyl-benzimidazolyl-carbocyanine iodide (JC-1) does distinguish between spermatozoa with poorly and highly functional mitochondria. 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 IVF rates were higher in the high potential group than in the low potential group [73, 74].

Acrosomal integrity

Acrosomal reaction, induced or spontaneous, is associated with surface membrane changes of specific glycoproteins. Pisum sativum agglutinin (PSA) is a lectin from the pea plant that binds to the acrosomal matrix and can stain acrosome reacted spermatozoa. Arachis hypogaea agglutinin (PNA) is a lectin from the peanut plant that binds to the outer acrosomal membrane of fixed spermatozoa, indicating acrosome-intact cells [75, 76]. Flow cytometry can assess acrosomal reaction changes using fluorescently labeled lectins that bind specifically to their receptors or by using specific anti-CD46 monoclonal antibody. Herrera et al. reported the incidence of a progesterone-induced acrosome reaction was significantly lower in subfertile (5.75%) compared with fertile boars (10.0%), suggesting that assessment of the induced acrosome reaction may be a useful parameter to assess fertility [77].

Another method of detecting acrosomal integrity is with LysoTracker®, which is specific for acidotropic organelles, such as the acrosome, and has been used to indicate acrosome-intact bovine spermatozoa. In another study, Thomas et al. used LysoTracker® to compare the functional status of bovine spermatozoa that had been stored at 5-8°C for 24 h or cryopreserved [78, 79]. Differences in acrosome integrity were noted between bull spermatozoa stored for 24 h but not those that were cryopreserved, suggesting that the freezing process results in a more uniform status of sperm organelles than in unfrozen samples.

Sperm capacitation status

Capacitation status has been assessed via calcium-mediated changes using the fluorescent antibiotic, CTC. The CTC-calcium complex preferentially binds to hydrophobic regions, such as the cell membrane, resulting in staining patterns characteristic of non-capacitated (F-), capacitated (B-) and acrosome-reacted (AR-pattern) spermatozoa. Observations of CTC staining patterns have been performed routinely using fluorescence microscopy and have been adapted for the flow cytometer by Maxwell and Johnson [80]. Using the flow cytometer, they found that incubation (38°C, 4 h), flow sorting, cooling (to 15 or 5°C), and freezing reduced the proportion of F-pattern and live spermatozoa and increased the proportion of B-, AR-pattern, and dead spermatozoa in comparison with fresh boar spermatozoa [11, 81].

Another way to assess sperm capacitation is with the hydrophobic dye Merocynine 540, which is believed to detect a decreased packing order of phospholipids in the outer leaflet of the plasma membrane lipid bilayer seen in capacitated spermatozoa [60, 82]. In the pig, bicarbonate brings an increase in plasma membrane lipid asymmetry, and an increase in the ability of live acrosome-intact cells to bind zona pellucida components. Under these conditions, M540 staining is also increased [83-85].

Lipid peroxidation

Lipid peroxidation can be followed, quantified, and localized after labeling sperm with lipid fluorochrome such as C_{11} BODIPY$^{581/591}$. This probe fluoresces red if intact, but it fluoresces green to orange if oxidized by ROS or peroxynitrate, respectively. The degree of probe peroxidation can be followed in particular sperm subpopulations using FCM or localized in each spermatozoon using life cell imaging microscopy [86].

Intracellular reactive oxygen species detection

Oxidative stress (OS) is a well known cause for male infertility. Measurement of ROS is critical for early detection of OS. Measurement of the specific ROS provides more sensitive markers for the OS. The Cleveland Clinic Andrology Laboratory has standardized an assay for simultaneous measurement of both intracellular H_2O_2 and $O_2^{-\bullet}$ in human spermatozoa [46]. Differential expression of intracellular H_2O_2 and $O_2^{-\bullet}$ was shown in sperm fractions.

Anti-sperm antibodies detection

Detection of sperm surface antibodies is important for infertility diagnosis and treatment. The use of FCM to detect sperm-bound antibodies and to quantitate the sperm antibody load (antibody molecules/spermatozoa) has been reported. After staining of washed sperm samples, dead sperm were excluded with FITC-conjugated fragments of anti-IgG and IgA antibodies by the use of calibration standards. The sperm load was 11,500±8,600 IgG molecules and 13,200±9,500 IgA molecules per spermatozoa. The sperm antibody load can be used to compare different patients or to follow up with the same patient. Flow cytometry has the potential reliability and objectivity to detect sperm antibodies [45, 87-89].

Poly (ADP-ribose) polymerase cleavage

Poly (ADP-ribose) polymerase (PARP) is a nuclear enzyme that helps in the DNA damage/repair process. DNA damage is the major stimulus for PARP activation. Poly (ADP-ribose) polymerase cleaved by activated caspase-3 stops the repair of extensively damaged DNA. Poly (ADP-ribose) polymerase homologues and cleaved PARP were shown in human spermatozoa by the intracellular staining protocol using FCM [47].

Detection of caspase activation

Caspases are complex proteins that are activated in the process of programmed cell death or apoptosis. The most important member is activated caspase-3, which plays a key role in apoptotic changes. Activated caspase-3, -7, or -9 could be detected by the intracellular staining protocol using FCM [90].

Sperm chromatin examination

Sperm chromatin is considered an independent marker for sperm quality. Fertile men show high percentages of sperm chromatin integrity and maturity. Many assays have been described for sperm chromatin assessment.

CMA3 is used as a fluorescent chromatin dye. CMA3 assay is a test for sperm maturity by FCM. It can stain the sperm with under-protamination. It is a useful diagnostic assay for male infertility that discriminates negative- from positive-stained spermatozoa with defective protamination. CMA3 can be interpreted using fluorescent microscopy or FCM [91-93].

The sperm chromatin structure assay (SCSA) is basically a FCM assay based upon abnormal sperm chromatin's high susceptibility to induced DNA denaturation [8]. The extent of DNA denaturation is determined by measuring the metachromatic shift of acridine orange (AO) from green fluorescence (intercalated into double-stranded nucleic acid, dsDNA) into red fluorescence (associated with single-stranded DNA, ssDNA) [94, 95]. Sperm chromatin structure assay results showed that the extent of DNA denaturation is expressed as the DNA fragmentation index (%DFI), which is the ratio of red to total fluorescence intensity, i.e. the level of damaged DNA over the total DNA. Sperm chromatin structure assay also can measure the fraction of cells with high DNA stainability (%HDS), representing immature spermatozoa with incomplete chromatin condensation [96, 97].

In the TUNEL assay, sperm are incubated with pro-proteases to provide access to DNA for labeled markers enzymatically added to 3'OH ends of broken DNA nicks. The TUNEL assay supposedly tags dsDNA breaks primarily which are lethal for any required gene. Labeled DNA can be detected by FCM [98-100]. However, subtle variations in the TUNEL technique, is a major concern as reported by a U.S. Environmental Protection Agency workshop review team [101].

Comparisons of data from TUNEL and SCSA measures have shown a 0.9 correlation between the two assays [99]. A disadvantage of TUNEL is that it cannot detect the immature sperm population, known as the HDS. In the SCSA, %HDS together with the %DFI was shown to be of importance for assessing male infertility [102] and reproductive toxic effects [103, 104]. Our group has overcome this by using PI with RNase as a counter-stain, so we get able to specify DNA binding and comment on the immature sperm fractions (unpublished data).

Telomere length and karyotyping

In a case report by Weissenberg, fluorescence in situ hybridization (FISH) was applied to identify normal, balanced, or imbalanced sperm cells of donor semen [105-107]. Flow cytometry can detect fluorescence karyotyping in spermatozoa and can detect the telomere length by flow-FISH technique in testicular tissue or ejaculated immature spermatozoa [108-110].

Sperm sorting

Sperm sorting from vaginal wash-collected cells with physiological solution and a flow cytometric reading of fresh semen is reported to increase the cost-benefit ratio in cases of rape [7, 111]. Other markers of spermatozoa used for research purposes such as selection of spermatozoa based on their positivity to annexin V binding or mitochondrial membrane potential.

The most common sorting is based on the haploid structure of the sperm with the advent of high resolution software, which makes possible differentiation between X- and Y- carrying spermatozoa. Recent efforts introduced this technology to select the Y-X sperm in the animal industry and in some endangered or exotic species and in humans for some genetic/familial disorders [53, 112, 113]. The ability to successively sort sperm must take into account the staining procedure, the relative susceptibility of sperm to laser exposure, high dilution, elevated pressure, and resistance to the several changes in media composition that occur during the sperm sex-sorting process [43, 112, 114-117].

Damage to sorted spermatozoa may occur during processing, staining, sorting, and/or cryopreservation. Unstained sorted sperm showed increased DNA damage by 2%, while stained sorted sperm showed an increase in DNA damage of 1.5%

[53, 118]. The mechanical stress and staining for sperm sorting as well as centrifugation increased the percentage of dead/damaged sperm by 18.6% [119-121]. Lowering the fluid pressure below 50 psi increased the survival of the sorted spermatozoa. Optimizing the sorting condition is required to achieve efficient acceptable sperm sorting yield [54, 112]. Ultra violet laser exposure and Hoechst staining tended to increase the incidence of chromosome aberrations [122], however, a recent study showed no genotoxic effects of sexing sperm found in cells resulting from pigs [123].

Conclusions

Flow cytometry applications allow objective, reproducible, and precise analysis of sufficient spermatozoa to provide for an accurate examination for semen specimen. Many fluorescent probes have been applied to indicate or predict sperm function. The question remains whether FCM assessments can be applied to routine investigations for male infertility or prediction of fertility in the field. To date, some single sperm parameters measured by FCM have been useful in differentiating sperm quality and fertility of individual males.

Flow cytometry may be an efficient tool that can provide the ideal help and solutions for many problems in the andrology lab and male infertility clinic. It also detects the fertilization and functional status of the spermatozoa by examining the DNA, acrosomal integrity, mitochondrial membrane function, and all other sperm sub-organelles. Flow cytometry sperm sorting may still need some development to maximize its benefits and safety. The current cost and potential applications of FCM still need evaluation for its usefulness in the management of infertile couples. However, recent developments that make FCM available for reasonable cost with acceptable quality may expand its availability in infertility clinics.

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