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Review

Flow cytometry and its use in modern human and veterinary andrology

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Abstract: Fertility of a male is sometimes difficult to assess and results obtained using regular sperm analysis methods are often inconclusive. Flow cytometry was proven to generate crucial information, that allows specialists to conclude upon the reproductive capacity of an individual. Together with computer assisted sperm analysis CASA systems, flow cytometers allow an in-depth analysis of fresh, chilled or frozen/thawed semen, which is currently essential for research purposes, but also for diagnosis of various male-related fertility disorders, both in veterinary and human medicine. Several parameters can be assessed using this method, such as sperm viability, acrosome integrity, mitochondrial activity, concentration and total sperm number, DNA fragmentation, capacitation, oxidative stress, etc. This paper provides a rapid reference to specialists involved in semen analysis by flow cytometry, regarding semen sample preparation, instrument setup, and evaluation of the most important sperm parameters (viability, acrosome reaction and mitochondrial activity).

Keywords: flow cytometry; mammalian sperm; viability; acrosome integrity; mitochondrial activity.

1. Introduction

Flow cytometers are made up of several independent systems that are interconnected to yield the final results, such as fluidics, that allows a single-stranded alignment of events, optics, made up of several lasers, light filters and detectors as well as electronics, which allows conversion of optical signals into electronic information which can be stored and analysed by a computer, equipped with a dedicated software. As such, flow cytometry represents a powerful technique that allows a complex and prompt evaluation, or even separation, of single cells (called events) found in suspension, making it therefore very suitable for sperm analysis. Cells can be thus evaluated regarding their size (forward scatter), internal complexity usually given by their granularity (side scatter) as well as fluorescent intensity (when stained with fluorescent antibodies or dyes that bind to the nucleus, cytoplasm, or membrane) [1]. Since fertility of a male is sometimes difficult to assess and results obtained using regular sperm analysis methods are often inconclusive, flow cytometry was proven to generate crucial information, that allows specialists to conclude upon the reproductive capacity of an individual. Several parameters can be assessed using this method, such as sperm viability, acrosome integrity, mitochondrial activity, concentration and total sperm number, DNA fragmentation, capacitation, oxidative stress, etc. [2]. This paper is aimed at providing a rapid reference to specialists involved in semen analysis by flow cytometry, regarding semen sample preparation, instrument setup, and evaluation of the most important sperm parameters (viability, acrosome reaction and mitochondrial activity).

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2. Semen Sample Preparation

Semen analyzed by flow cytometry may originate from fresh ejaculates, collected by any of the commonly used methods (masturbation, artificial vagina, electroejaculation, etc.) but may also be obtained after flushing of epididymis (from dead/slaughtered animals or following castration). Frozen semen samples may also be investigated by flow cytometry and such studies are of particular interest to assess efficacy of the freezing method or suitability of the extender used [3].

One of the biggest issues that has to be taken into consideration is interference between components of the seminal plasma or extender and staining dyes. In cases where this represents a serious concern, the most suitable method of sample clean-up or washing has to be employed. Usually, a density gradient centrifugation or the swim-up technique is adequate.

Another significant problem is related to the debris which is often present in the sperm extenders, due to the egg yolk or milk proteins. If debris is not removed or gated out, it can lead to false results as foreign particles may overlap unstained populations of spermatozoa [4]. On the other hand, there are dyes such as tetraethylbenzimidazolylcarbocyanine iodide (JC-1) which are lipophilic and therefore can bind to debris and yield misleading results. Gating out debris can be achieved on the FSC vs SSC dot plot, where it usually appears to have significantly lower FSC as spermatozoa.

Mathematical corrections are also possible but are time consuming and difficult to perform.

Another option is to use an intravital dye, such as Hoechst 33342 which only stains live cells, and therefore unstained events can be gated out as debris. The advantage of using this dye also resides in the fact that its fluorescent signal (UV range) does not overlap with any of the fluorochromes frequently used for semen analysis [5]. Nevertheless, the drawback is that the cytometer must have a violet laser or UV diode.

3. Instrument setup

In order to obtain accurate results, the flow cytometer must be checked and properly calibrated on a daily basis. Calibration microspheres are usually made of polystyrene latex and are labelled with fluorescent dyes. Each laboratory must establish a daily clean-up and calibration protocol, according to the type of equipment that is present and the instructions of the producer. Standardization of the procedure is very important, as it provides reproducibility, accuracy and reliability of results.

Next, the machine can be prepared for analysis, by choosing the optimum channels and optical filters needed, according to the type of experiment that is required. Usually, the blue 488 nm Argon ion laser at 488 nm is the only one that is needed, since most of the dyes used for sperm analysis emit green, orange or red fluorescence. Green fluorescence is read in FL1, orange in FL2 and red in FL3. Regarding filters, the 530/28 BP should be used for FL1, the 585/42 BP for FL2 and the 650 LP for FL3. All dot plots or histograms should be set to the logarithmic scale and signal height should be acquired for all parameters.

Compensation should always be performed, whenever multiple dyes are used to stain the same semen sample, in order to avoid fluorescence spill over and false results. This is usually achieved using compensation beads, that are stained with the same fluorochromes as those used in the experiment. Experienced users may also choose to perform manual compensation, after the samples are acquired.

Actual analysis begins by plotting forward scatter height (FSC-H) vs side scatter height (SSC-H) in order to define and gate the sperm population. This will also allow to remove from the gate any debris (mostly originating from the extenders) and also to eliminate any electronic noise. A total number of at least 10,000 events should be acquired, and, since the cells (spermatozoa) are small, the sample should be run at low speed for better accuracy.

4. Sperm viability assessment

DNA intercalating agents are fluorescent molecules, capable of passing through cellular membranes of cells and therefore stain the nuclei. Some of the molecules are able to pass through intact membranes and therefore also stain the nuclei of live cells. A frequently utilized green fluorescent dye, that stains all nuclei of spermatozoa (damaged or intact), is SYBR-14 (maximum emission at 516 nm) which is used in combination with a red dye - propidium iodide (PI, maximum emission at 617 nm). The latter is also an intercalating agent which can only penetrate the damaged plasma membrane and therefore stains the nucleus of only dead spermatozoa. PI signal quenches SYBR-14 fluorescence, thus live spermatozoa are stained in green while dead spermatozoa are red [6]. Nowadays, there are several live/dead kits available on the marked, which allow differentiation between live and dead spermatozoa. Those kits also permit an easy

discrimination between spermatozoa and debris, and therefore eliminate the need of performing additional staining steps.

These kits usually contain a 1 mM solution of SYBR-14 in DMSO and a 2.4 mM solution of PI in water. A 20 μM SYBR-14 stock solution in DMSO is initially prepared, which can be stored frozen. When needed, the working solution is made by adding 5 μL of the 20 μM SYBR-14 solution and 50 μL of the 2.4 mM PI solution to 10 ml of buffer, such as HEPES for approximately 20 samples.

Semen samples can be successfully stained with this working solution, although the manufacturers recommend separate incubations. Spermatozoa should be diluted to a concentration of 1-2x106/ml in 0.5 ml staining solution, in cytometry tubes. Incubation should be made in the dark at 37°C and run in the cytometer right away. Two dot plots are needed for each tube, one for spermatozoa gating (FSC-H vs SSC-H) and another one for viability assessment (FL1-H vs FL3-H). Following the analysis, 3 distinct populations are visible on the dot plot:

- SYBR-14+/PI– (live spermatozoa);
- SYBR-14+/PI+ (moribund spermatozoa);
- SYBR-14–/PI+ (dead spermatozoa).

The SYBR-14–/PI– events are likely to represent debris which should be gated out.

Alternatively, sperm viability can be assessed using a combination of 3 fluorescent dyes - SNARF-1, YO-PRO-1 and ethidium homodimer. Thus, 4 subpopulations of spermatozoa can be detected: one viable, with stable membranes (SNARF-1+), and three with compromised membranes: YO-PRO-1+/Eth-, YO-PRO-1-/Eth+ and YO-PRO-1+/Eth+ [7,8].

5. Evaluation of acrosome reaction

The acrosome is a structure that covers the anterior part of spermatozoa in mammals and contains enzymes that allow penetration of the zona pellucida during fertilization. Semen cryopreservation sometimes induces a so-called acrosome reaction, which means inactivation of the specific acrosomal enzymes, which renders spermatozoa inefficient [9]. Acrosome integrity can be assessed using FITC or PE labelled plant lectins (pea agglutinin-PSA or peanut agglutinin-PNA). PSA cannot breach the membrane of an intact acrosome and thus, if stained, spermatozoa are judged as damaged. In practice, PNA is preferred to PSA as the latter was demonstrated to non-specifically bind to the egg-yolk found in semen extenders as well as to other fragments of spermatozoa [10].

To prepare the stock solution, lyophilized PNA-FITC is resuspended in water to a concentration of 0.1-1 mg/ml and then stored frozen until use. The 1 mg/ml solution is usually preferred. If storage at the refrigeration temperature is needed, a supplementation with 2 mM Na-azide is mandatory.

The usual protocol when assessing the acrosome reaction is to combine PNA-FITC staining with PI, in order to also observe the population of non-viable spermatozoa. The PI stock solution can also be stored frozen, at a concentration between 50 μg-5mg/ml, but most frequently a 1 mg/ml solution is preferred.

The working solutions can easily be prepared based on the stock solutions at 1 mg/ml. After thawing, 10 μl of both the PNA-FITC and PI solutions are added to 10 ml of PBS and 0.5 ml of the resulting solution are used to dilute the semen sample to a concentration of 1-2 x 106 spermatozoa/ml. The working solution should be kept in the darkness until use, but not more than 12-24 hours. After staining, spermatozoa should be incubated in darkness for 15 minutes at 37°C.

Next, the samples are run in the flow cytometer and two dot plots are needed: one for spermatozoa gating (FSC-H vs SSC-H) and another for acrosome reaction/viability (FL1-H vs FL3-H).

Following analysis, four different populations of spermatozoa can be identified:

- the PNA-FITC–/PI– population is represented by viable spermatozoa with unreacted acrosome;
- the PNA-FITC+/PI– population is represented by viable spermatozoa with reacted acrosome;
- the PNA-FITC+/PI+ population is represented by moribund or dead spermatozoa with reacted acrosome;
- the PNA-FITC–/PI+ population is represented by moribund or dead spermatozoa with unreacted acrosome.

6. Evaluation of mitochondrial activity

In mammalian spermatozoa, mitochondria are essential organelles which play a crucial role for their motility and fertilizing capability, by contributing to ATP and reactive oxygen species production as well

as calcium level control. Their integrity and activity can be assessed by quantitatively evaluating their membrane potential, using a fluorescent dye called 5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimi-dazoylcarbocyanine iodide (JC-1). When mitochondrial membrane potential is high, JC-1 forms combinations that produce red fluorescence, while in the case of low mitochondrial membrane potential, JC-1 stays monomeric and emits green fluorescence (11).

The JC-1 stock solution can be prepared by resuspending the lyophilized powder in DMSO, to a concentration of 2 mg/ml (3 mM) and stored frozen in a dark vial. The working solution is a 1000 fold dilution of the stock solution. Therefore, 10 µl of the stock solution should be added to 10 ml of PBS and 0.5 ml of the resulting solution are used to dilute the semen sample to a concentration of 1-2 x 106 spermatozoa/ml. Following incubation for 15-20 minutes in darkness at 37°C, samples are run in the flow cytometer and visualized in two dot plots: one for gating the spermatozoa (the usual FSC-H vs SSC-H) and another for mitochondrial membrane potential (FL1-H vs FL2-H).

Analysis of dot plots allows classification of ejaculates according to mitochondrial activity of spermatozoa, as follows:

- spermatozoa with high FL1-H and low FL2-H have a low mitochondrial membrane potential and are considered of low fertilizing ability;
- spermatozoa with low FL1-H and high FL2-H have high mitochondrial membrane potential and potentially poses a fertilizing ability (this category should be the most abundant in good quality ejaculates);
- spermatozoa with high FL1-H and high or moderate FL2-H are considered to have large gaps in their mitochondria and are therefore of lower quality;
- spermatozoa with low FL1-H and low FL2-H are likely dead and have a damaged midpiece.

7. Conclusions

Flow cytometry is an extremely useful and powerful tool that can be used for advanced semen analysis as it provides quick and reliable results, enabling an accurate estimation of various semen parameters.

Together with computer assisted sperm analysis CASA systems, flow cytometers allow an in-depth analysis of fresh, chilled or frozen/thawed semen, which is currently essential for research purposes, but also for diagnosis of various male-related fertility disorders, both in veterinary and human medicine.

The equipment required is indeed quite expensive and the operators need special training, while experience is also an asset.

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