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Review Article

Flow Cytometry Probes to Evaluate Stallion Spermatozoa

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ABSTRACT

The aim of this review is to present the current probes available that assess different compartments and functions of stallion spermatozoa, including assays to investigate the functionality of the membranes, nucleus, and mitochondria. New multiparametric protocols for the assessment of stallion sperm, recently developed in the laboratory of the authors, will also be presented. The potential clinical applicability of a diagnostic test based on flow cytometry will also be discussed.

1. Introduction

This century, and particularly the last decade, has been witness to intensive research in sperm biology. Consequently, a better understanding of sperm function in relation to clinical andrology and sperm biotechnologies [1,2] has arisen. Relevant advances in stallion sperm biology include, among others, the following: the understanding of osmotic shock [3–5] and its implications on cryopreservation, which promoted the development of new protocols based on more permeant cryoprotectants [6–10]; advances in understanding the role of reactive oxygen species (ROS) [11–14]; and the development of practical methods for stallion sperm separation and selection through colloidal centrifugation [15–21]. More recently, the understanding of sperm bioenergetics and mitochondrial functionality has become two hot topics in stallion andrology. Beside these advances, better tools for sperm assessment have been developed, in which flow cytometry has played a major role. The aim of this review is to present a rapid summary of the current probes available to assess stallion sperm and describe new protocols for the assessment of stallion sperm, including those recently developed in the laboratory of the authors. The potential clinical applicability of a diagnostic test based on flow cytometry will also be discussed. Interestingly, these assays have been recently supported with field fertility data [22].

2. Basic Principles of Flow Cytometry Applied to Sperm Analysis

Flow cytometry is an analytical technology that simultaneously measures and analyses multiple physical characteristics of single spermatozoon. The properties measured include the size of the spermatozoa in the forward scatter detector, the complexity in the side scatter detector, and the relative fluorescence intensity in fluorescence detectors (FL). These characteristics are detected using a fluidic and optical to electronic coupling system that records how each individual spermatozoon or other particle presents in the sample, scatters incident laser light, and emits fluorescence. In the flow cytometer, spermatozoa are carried to the laser interrogation point in

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a fluid stream (sheath fluid). When they pass through the laser intercept, they scatter laser light, and any fluorescent molecules present are excited and emit light in different wavelengths. Appropriately positioned lenses collect the scatter and fluorescent light. A combination of beam splitters and filters steer fluorescence to detectors that produce electronic signals proportional to the optical signals striking them. List mode data are collected on every single spermatozoon and stored in the computer; these data are analyzed and provide information about subpopulations in the sample and are displayed graphically in histograms and dot plots. Fluorescent compounds are used to study stallion sperm functionality. A fluorescent compound absorbs light energy over a range of wavelengths characteristic for each. This absorption of light causes an electron in the fluorescent compound to be raised to a higher energy level; the electron quickly returns to the ground state, releasing the excess energy as a photon. This transition of the energy is termed fluorescence. The range of wavelengths in which a fluorescent compound can be excited is called absorption spectrum, whereas the range of wavelengths emitted is called emission spectrum. Ideally, the light produced by emission should be different from the light used for excitation, and this difference is known as the Stokes shift. The wavelength of emission is longer than the wavelength of excitation because typically more energy is used to excite the electrons of the fluorochrome than the energy released (as light) when the electrons return to the resting state. For example, a commonly used fluorochrome, fluorescein isothiocianate, absorbs light in the range 400–550 nm, with a peak or maximum excitation at 490 nm (the laser is used to excite a particular dye, the blue laser [488 nm] in this case), and emits in the range 475–700 nm, with a peak at 525 nm (green spectrum). This range of wavelengths determines the filters and the channels (fluorescence channels FL_) of detection to be used. Combining different fluorochromes with multiple wavelengths of excitation and emission allows multiple and simultaneous measurements; however, compensation for spectral overlap has to be considered and carefully managed. When two or more dyes are used simultaneously, there is a chance that their emission profiles will coincide, making measurement of the true fluorescence for each one difficult. This outcome can be avoided by using dyes at distant positions in the spectrum; for example, a dye that is excited with the violet laser (405 nm) and a dye excited with the red laser (647 nm). However, using dyes at distant positions is not always possible, and a process called fluorescence compensation is applied. This process calculates how much interference, as a percentage, a fluorochrome will have in a channel that was not assigned specifically to measure it. The design of an experiment in the flow cytometer implies careful selection of probes suitable for each particular cytometer, identification of potential spectral overlap among probes, use of proper controls for positive and negative populations (unstained sample), and controls for compensation (single-stained samples in which there is a stained and unstained population for each dye to be used in the experiment). Depending on each particular experiment, other controls can be necessary, including fluorescence minus one controls, isotype controls, or secondary antibody only controls.

3. The Sperm Membrane: Integrity, Permeability, Fluidity, and Functionality

Traditional assessment of the sperm membrane has focused on the physical integrity using dye exclusion tests. Classical combinations of fluorescent probes for this purpose include the combination of SYBR-14 and propidium iodide (PI) (http://tools.thermofisher.com/content/sfs/manuals/mp07011.pdf). This combination is marketed as a kit, and detailed instructions for use are provided by the manufacturer. This combination of probes uses the blue laser for excitation (488 nm) and provides two wavelengths of emission: green for live sperm (521 nm SYBR-14) and red for dead sperm (635 nm PI). This combination of probes allows the rapid discrimination of debris (because both are DNA binding probes). Spillover between emission wavelengths of both probes can occur (521 nM for SYBR-14 and 635 nM for PI), and proper fluorescence compensation has to be established to use this probe pair because SYBR-14 still has, on average, a 6% emission at 635 nM. Furthermore, staining with SYBR-14/PI discriminates only between live/dead sperm and does not expose states of membrane damage. In addition, doublets have to be identified to correctly interpret this assay. Alternatively, Hoechst 33342 and PI [23] also allow the rapid discrimination of debris and have the advantage that spillover is unlikely due to the distinct excitation and emission spectra of these probes. However, H33342 needs a violet or ultraviolet laser for excitation. Detection of more subtle changes in the sperm membrane requires the use of other probes. Fluidity of sperm membranes can be assessed with merocyanine 540 [24], and subtle increases in the permeability of the sperm plasma membrane can be detected with YoPro-1 [25,26]. Yo-Pro-1 is routinely used in the authors' laboratory in combination with PI; H33342 is also incorporated to sort debris. These combinations allow the detection of changes in sperm membranes at much earlier stages than SYBR-14 and correlate better with motility and sperm velocities [26].

Recently, new fixable fluorescent dyes have become available in multiple colors, which facilitate experiments with multiple spectra in fixed sperm. These probes are based on the reaction of fluorescent reactive dye with cellular amines. These are proprietary dyes that can permeate the compromised membranes of necrotic cells and react with free amines both in the cytoplasm and on the cell surface, resulting in intense fluorescent staining. In contrast, only the cell surface amines of intact cells are available to react with the dye, resulting in relatively dim staining. The discrimination is maintained following formalin fixation of the sample under conditions that inactivate pathogens. Moreover, these assays use only one channel of the flow cytometer, leaving the other channels available for multicolor panels. The potential advantage of these dyes is the ability to process and stain the samples at locations remote to the flow cytometer.

The evaluation of the sperm's ability to undergo the acrosome reaction in response to an agonist challenge is useful in cases of infertility in certain thoroughbred lines.