Anim. Reprod., v.12, n.3, p.351-355, Jul./Sept. 2015

Multiparametric flow cytometry: a relevant tool for sperm function evaluation

F.J. Peña[1]

Laboratory of Equine Reproduction and Spermatology, Veterinary Teaching Hospital, University of Extremadura, Cáceres, Spain.

Abstract

Nowadays in human and animal andrology flow cytometry is recognized as a robust tool for the evaluation of sperm quality and function. However, in this particular field, this technique has not reached the sophistication of other areas of biology and medicine. In recent years more sophisticated flow cytometers are being introduced in andrology laboratories, and the number of tests that can be potentially used in the evaluation of the sperm physiology has increased accordingly. In this review recent advances in the evaluation of sperm will be discussed; representing new techniques in flow cytometry, many of them able to measure simultaneously, in a single test, different degrees of damage in different sperm regions and/or changes in functionality.

Keywords: multiparametric flow cytometry, sperm flow cytometry.

Introduction

The ultimate goal of semen analysis is to determine the fertility of a sire; depending of individual value of each sire, semen analysis may determine whether a sire is simply eliminated as a semen donor, or will receive treatment to improve his fertility. While the definitive proof of fertility is the offspring born from the sire, robust laboratory test are needed. Fertility trials are expensive and time consuming, moreover while in particular species such as pigs and bulls can be relatively easy to perform, in other species this may result much more complicated. Understanding sperm function is an absolute pre-requisite for fertility evaluation. Spermatozoa are terminal fully differentiated cells, with functions that ought to be expressed at specific time intervals, and also have to be able to support dramatic changes in their environment in their journey from the tail of the epididymis to the oviduct. Moreover must have the capacity to readily respond to specific signals originated in the female genitalia. In a landmark paper by Amann and Hammerstedt (1993) the complexity of sperm evaluation was clearly underlined, and addressed the need to evaluate the multiple functions and attributes that the spermatozoa must fulfill to reach and fertilize an oocyte. More recently the discovery of the heterogeneous nature of the ejaculate may introduce the need to develop methods to identify the fertilizing population within a given sample. Classical sperm evaluation implies in most circumstances the evaluation of single attributes, i.e. morphology, sperm number, subjective motility. More recent developments, like CASA evaluates attributes such as sperm velocities that shall depend of the correct function of different sperm attributes, i.e. adequate source of energy and fully functional metabolic pathways. However evaluation of multiple attributes with a single or few tests is not common practice yet.

Flow cytometry in sperm evaluation

One important aspect of sperm evaluation is to born in mind the representativeness of the sample analyzed with respect of the whole ejaculate. In this respect, has been determined that several thousands of spermatozoa is preferred for the sperm analysis, unfortunately conventional semen analysis usually evaluates a few hundred sperm, and even more elaborated analysis, involving fluorescence microscopy and computer assisted sperm analysis, share the same limitation. Flow cytometry was introduced in semen analysis back in the late 70s early 80s. When introducing the terms “flow cytometry and sperm” in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) 1839 papers result, with the first two ones published in 1977 (van Dilla *et al*., 1977) and 1978 (Meistrich *et al*., 1978). Flow cytometry allows a better representation of the sample analyzed, at least numerically, since with this technique is easy to evaluate many thousands of spermatozoa in a few seconds. Since the early use of flow cytometry for DNA analysis, fluorescent methods were introduced in the 90s to monitor membrane integrity, and more recently mitochondrial membrane potential, oxidative status, membrane fluidity and permeability, lipid peroxidation, and tyrosine phosphorylation of sperm proteins, among other assays, have been developed (Peña *et al*., 2005b; Peña, 2007; Ortega Ferrusola *et al*., 2009a, b). Recent reviews (Martinez Pastor *et al*., 2010; Petrunkina and Harrison, 2011, 2013) have addressed flow cytometry techniques currently in use in veterinary andrology, and the reader is referred to them; here we will focus in new possibilities that flow cytometry offer for the simultaneous evaluation of different sperm compartments and functions.

Multiparametric flow cytometry

Multicolor analytical approaches are widely used in different fields of medicine and biology. If these procedures could be adapted to sperm assessment, multiple attributes on individual sperm cells could be rapidly evaluated. This will also introduce a solution to one the major drawbacks that historically has had sperm

[1]Corresponding author: fjuanpvega@unex.es
Received: May25, 2015
Accepted: July 7, 2015

evaluation, the problem of not being able to test multiple attributes simultaneously at the single cell level. Two to three lasers systems have become more affordable, and now are entering in the andrology laboratories. It has been proposed that this systems would allow simultaneous assessment of all major parameters covering the route to fertilization provided that careful selection of probes, correct optical configuration and accurate multicolor compensation are introduced (Petrunkina and Harrison, 2011). Equipment having three excitation lasers has been recently incorporated to the laboratory of the author (MACSQuant Analyzer 10, Miltenyi, Biotech, Bergisch Gladbach, Germany). This is a compact flow cytometer equipped with violet (Excitation wavelength 405 nm), blue (Excitation wavelength 488 nm) and red (Excitation wavelength 635 nm) lasers, and 8 photomultiplier tubes plus FSC and SCC detectors. The excitation and emission wave lengths that this system provides, allows the use of combinations of probes that can assess simultaneously multiple parameters in a large number of spermatozoa (usually >40,000). Two panels (and combinations) are now routinely used in our laboratory to measure simultaneously membrane integrity, functional changes in membrane permeability and mitochondrial membrane potential or oxidative stress, and at the same time gate out debris easily. Other panels are designed to determine simultaneously membrane integrity, caspase activity and mitochondrial membrane potential or oxidative stress. The first panel involves the use of Hoechst 33342, propidium iodide or ethidium homodimer, YoPro-1 and Mitotracker deep Red or CellRox Deep Red reagent. Hoechst 33342 can be excited with the violet laser, YoPro-1 and ethidium homodimer are excited with the blue laser and Mitotracker deep red is excited with the red laser. Correct compensation for spectral overlap is critical in multicolor protocols; unstained and single stained controls are mandatory. The first advantage of this combination of probes is that "alien" particles can be easily gated out from the analysis. Only Hoechst 33342 positive events are considered spermatozoa, avoiding errors inherent to gating decisions based in FSS and SCC characteristics. Multiple dot plots can be created, a dot plot combining Hoechst 33342 with propidium iodide or Ethidium homodimer can be used to determine the percentage of live and dead spermatozoa in the sample, being an alternative to the traditional use of SYBR-14/PI. Combinations of YoPro-1/propidium iodide and Hoechst 33342/YoPro-1 can be used to detect changes in membrane permeability; these variations may be related either to early sperm damage or being capacitation related changes. Finally mitochondrial membrane potential is assessed with Mitotracker deep Red, or oxidative stress assessed with Cell Rox Deep red reagent. Hierarchical gating can be applied to determine, for example, sources of reactive oxygen species (ROS) in specific sperm populations. An example of one of these panels is given in Fig. 1.

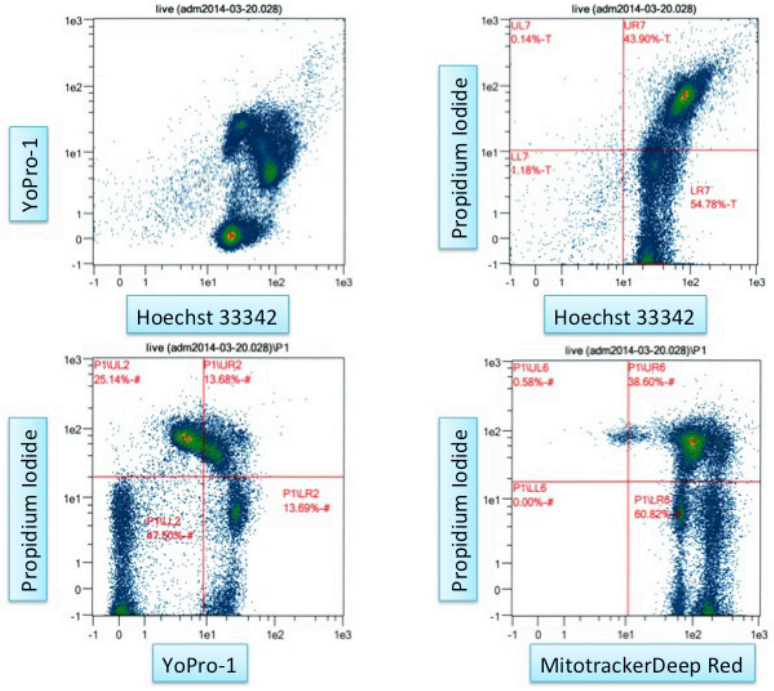

Figure 1. Example of a multicolor panel for simultaneous evaluation of different sperm parameters. Spermatozoa are stained with Hoechst 33342, YoPro-1, Propidium Iodide and Mitotracker deep Red. In the upper right dot plot information of the percentage of live spermatozoa are given thanks to the combination of Hoechst 33342, that identifies DNA bearing particles (spermatozoa) and Propidium Iodide (PI) that only stains dead sperm. Changes in membrane permeability are detected with YoPro-1 in combination either with PI or Hoechst allowing to detect spermatozoa in a early stage of membrane destabilization, finally the percentage of spermatozoa depicting active mitochondria are detected using Mitotracker deep red.

Additional markers can be incorporated to these panels, for example specific antiphosphotyrosine antibodies labeled with krome orange or pacific orange and detection in the V2 channel (405/525-50) and/or a calcium yellow sensor (488/585-40) can be incorporated to be detected in the B2 channel to asses sperm capacitation.

This panel only represents an example of the multiple combinations that multiparametric flow cytometry offers. Potential combinations of fluorophores and panels have been recently published (Petrunkina and Harrison, 2013)

Fixable dyes

Recently new fixable dyes have become available in the market (www.lifetechnologies.com). These dyes are available in multiple colors easily allowing multicolor experiments. These assays are based on the reaction of a fluorescent reactive dye with cellular amines. The reactive dye can permeate the compromised membranes of the necrotic cells and react with free amines both in the interior and on the cell's surface resulting in intense fluorescent staining. In contrast only the cell surface amines of the intact cells are available to react with the dye resulting in relatively dim staining.

The discrimination is completely preserved following fixation of the sample by formaldehyde under conditions that inactivate pathogens. Moreover this assays use only one channel of the flow cytometer leaving the other channels available for multicolor panels.

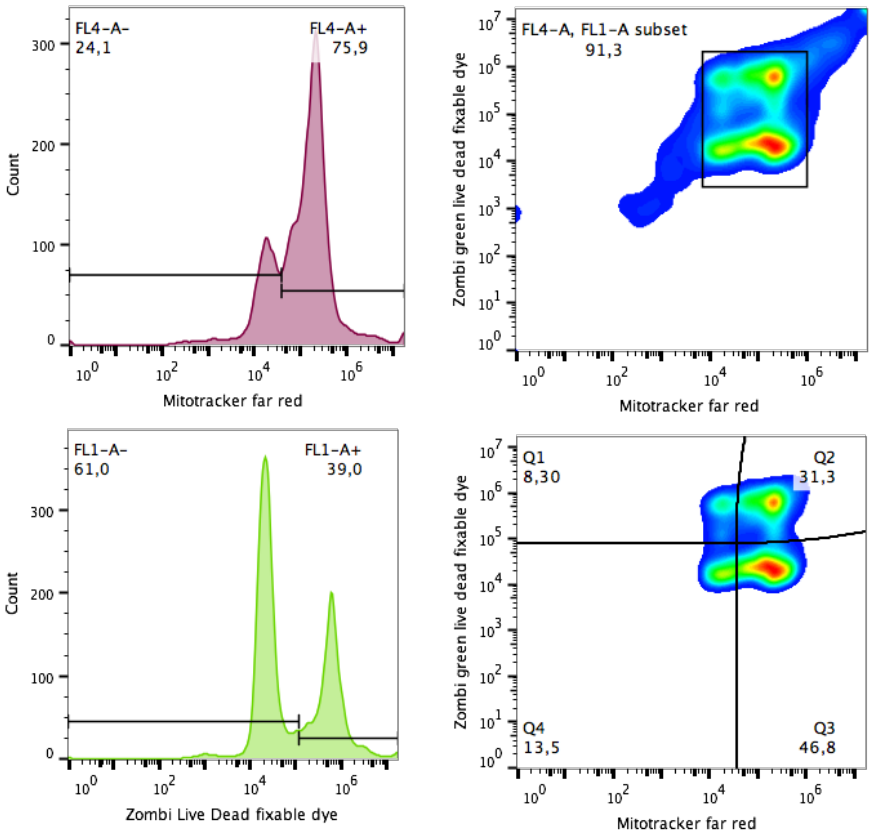

Figure 2. Example of a fixable dye protocol, sperm was stained with Live Dead fixable dye and mitotracker deep red. This assay allows to determine the subpopulation of live spermatozoa, with high mitochondrial membrane potential, represented in Q2.

Cell sorting; not only for sexing?

The theory of the existence of sperm subpopulations within the mammalian ejaculate is gaining growing consensus within the scientific community (Abaigar *et al*., 1999, 2001; Quintero-Moreno *et al*., 2003; Martinez *et al*., 2006). The mammalian ejaculate is a heterogeneous group of different sperm subpopulations showing different responses to physiological or biotechnological stimuli (Abaigar *et al*., 1999; Martinez-Pastor *et al*., 2005), motility patterns (Quintero-Moreno *et al*., 2003, 2004; Ortega-Ferrusola *et al*., 2009c) and even different morphometric characteristics (Thurston *et al*., 2001; Nunez-Martinez *et al*., 2005, 2007; Peña *et al*., 2005a). In many species semen is ejaculated in fractions, where spermatozoa are embedded in the secretions of the male accessory glands, in variable numbers, with variable amounts of fluid and, probably proteins, in each fraction (Rodriguez-Martinez *et al*., 2008). This heterogeneity in

the composition of seminal plasma affects sperm function, as has been demonstrated *in vitro* (Caballero *et al*., 2004; Rodriguez-Martinez *et al*., 2008; Saravia *et al*., 2009). However once the ejaculate is deposited in the female genital tract, the spermatozoa losses contact with the seminal plasma, and are exposed to different environments within the female genitalia. Using the boar species as model, and in experiments performing heterospermic inseminations (Satake *et al*., 2006), the role of the presence of sperm subpopulations in sperm selection has been disclosed. When spermatozoa from two or more boars are mixed and females inseminated the resulting litters are skewed in favor of one male. Bicarbonate responsiveness varies among sperm subpopulations between males, and specific oviductal proteins modulate this response. This mechanism selects sperm subpopulations to reach the oocytes for fertilization. All these scientific evidences provide an explanation of why the overall correlation with fertility is usually low when laboratory test of sperm function that ignore the sperm subpopulation structure are performed (Rodriguez-Martinez, 2003; Holt and Van Look, 2004). One of the potential roles of this sperm subpopulation structure may be a mechanism to help the fertilizing subpopulation of spermatozoa to avoid the immune response of the female while the immune system focus in redundant sperm. Recent research suggests that spermatozoa presenting phosphatidylserine (PS) translocation are preferentially phagocytized; in this way intact spermatozoa have greater possibilities to reach the oocyte for fertilization. Based in the assumption of the existence of a specific fertilizing group of spermatozoa in the ejaculate, recent studies have tried to identify this specific subpopulation and develop techniques for its selection based in flow cytometry (Sousa *et al*., 2011; Ribeiro *et al*., 2013). Sousa *et al*. (2011) used as sorting criteria the spermatozoa showing active mitochondria, and found that this defined a more functional population, with lower chromatin damage and sperm more able to decondense and participate in early embryo development. Furthermore flow cytometry cell sorting was better that traditional swim up for sperm selection. In another experiment Ribeiro *et al.* (2013) used the YoPro-1 staining as sorting criteria, the subpopulation selected was YoPro-1 negative spermatozoa. Using this approach dead and apoptotic spermatozoa were removed, and compared with traditional swim-up techniques the sorted viable population showed a significantly reduced population of spermatozoa with fragmented DNA. Although these techniques may be difficult to implement in production animal practices, may deserve consideration in cases of subfertility in animals of high individual value.

Concluding remarks

Flow cytometry has been in use for sperm analysis from the early 90s, first as a research tool and later introduced in large AI centers; initially most flow cytometers used a single source of excitation (normally a blue laser). More recently more sophisticated equipment has become available in veterinary andrology labs allowing multiple sources of excitation and detection of a large number of parameters. The development of assays that can evaluate simultaneously multiple sperm compartments and functions in a large number of cells shall improve sperm evaluation and establish better correlations with field fertility.

Acknowledgments

The author received financial support for studies reported in this review from Ministerio de Economía y Competitividad-FEDER in Madrid, Spain grant AGL 2013-43211-R, and Gobierno de Extremadura-FEDER, grants (GR 10010 and PCE1002).

References

Abaigar T, Holt WV, Harrison RA, del Barrio G. 1999. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. *Biol Reprod*, 60:32-41.

Abaigar T, Cano M, Pickard AR, Holt WV. 2001. Use of computer-assisted sperm motility assessment and multivariate pattern analysis to characterize ejaculate quality in Mohor gazelles (*Gazella dama mhorr*): effects of body weight, electroejaculation technique and short-term semen storage. *Reproduction*, 122:265-273.

Amann RP, Hammerstedt RH. 1993. In vitro evaluation of sperm quality: an opinion. *J Androl*, 14:397-406.

Caballero I, Vazquez JM, Centurion F, Rodriguez-Martinez H, Parrilla I, Roca J, Cuello C, Martinez EA. 2004. Comparative effects of autologous and homologous seminal plasma on the viability of largely extended boar spermatozoa. *Reprod Domest Anim*, 39:370-375.

Holt WV, Van Look KJ. 2004. Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. *Reproduction*, 127:527-535.

Martinez IN, Moran JM, Pena FJ. 2006. Two-step cluster procedure after principal component analysis identifies sperm subpopulations in canine ejaculates and its relation to cryoresistance. *J Androl*, 27:596-603.

Martinez-Pastor F, Garcia-Macias V, Alvarez M, Herraez P, Anel L, de Paz P. 2005. Sperm subpopulations in Iberian red deer epididymal sperm and their changes through the cryopreservation process. *Biol Reprod*, 72:316-327.

Martinez Pastor F, Mata Campuzano M, Alvarez Rodriguez M, Alvarez M, Anel L, de Paz P. 2010. Probes and techniques for sperm evaluation by flow cytometry. *Reprod Domest Anim*, 45(suppl. 2):67-78.

Meistrich ML, Lake S, Steinmetz LL, Gledhill BL. 1978. Flow cytometry of DNA in mouse sperm and testis nuclei. *Mutat Res*, 49:383-396

Nunez-Martinez I, Moran JM, Pena FJ. 2005. Do computer-assisted, morphometric-derived sperm characteristics reflect DNA status in canine spermatozoa? *Reprod Domest Anim*, 40:537-543.

Nunez-Martinez I, Moran JM, Pena FJ. 2007. Sperm indexes obtained using computer-assisted morphometry provide a forecast of the freezability of canine sperm. *Int J Androl*, 30:182-189.

Ortega Ferrusola C, González Fernández L, García Macías B, Salazar Sandoval C, Morell JM, Rodríguez Martinez H, Tapia JA, Peña FJ. 2009a. Lipid peroxidation, assessed with BODIPY-C11, increases after cryopreservation of stallion spermatozoa is stallion dependent and relates to "apoptosis like" changes. *Reproduction*, 138:55-63.

Ortega Ferrusola C, González Fernández L, García Macías B, Salazar Sandoval C, Morillo Rodríguez A, Rodríguez Martinez H, Tapia JA, Peña FJ. 2009b. Effect of cryopreservation on nitric oxide production in stallion spermatozoa. *Biol Reprod*, 81:1106-1111.

Ortega-Ferrusola C, Macias Garcia B, Suarez Rama V, Gallardo-Bolanos JM, Gonzalez-Fernandez L, Tapia JA, Rodríguez-Martinez H, Peña FJ. 2009c. Identification of sperm subpopulations in stallion ejaculates: changes after cryopreservation and comparison with traditional statistics. *Reprod Domest Anim*, 44:419-423.

Peña FJ, Saravia F, Garcia-Herreros M, Nunez-martinez I, Tapia JA, Johannisson A, Wallgren M, Rodríguez-Martínez H. 2005a. Identification of sperm morphometric subpopulations in two different portions of the boar ejaculate and its relation to postthaw quality. *J Androl*, 26:716-723.

Peña FJ, Saravia F, Johannison A, Wallgren M, Rodriguez Martinez H. 2005b. A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa. *Int J Androl*, 28:107-114.

Peña FJ. 2007. Detecting subtle changes in sperm membranes in veterinary andrology. *Asian J Androl,* 9:731-737.

Petrunkina AM, Harrison RAP. 2011. Cytometric solutions in veterinary andrology: developments advantages, and limitations. *Cytometry Part A*, 79A:338-348

Petrunkina AM, Harrison RAP. 2013. Fluorescence technologies for evaluating male gamete (dys)function. *Reprod Domest Anim*, 48 (suppl. 1):11-14

Quintero-Moreno A, Miro J, Teresa Rigau A, Rodriguez-Gil JE. 2003. Identification of sperm subpopulations with specific motility characteristics in stallion ejaculates. *Theriogenology*, 59:1973-1990.

Quintero-Moreno A, Rigau T, Rodriguez-Gil JE. 2004. Regression analyses and motile sperm subpopulation structure study as improving tools in boar semen quality analysis. *Theriogenology*, 61:673-690.

Ribeiro SC, Sartorius G, Pletscher F, de Geyter M, Zhang H, de Geyter C. 2013. Isolation of spermatozoa with low levels of fragmented DNA with the use of flow cytometry and sorting. *Fertil Steril*, 100:686-694

Rodriguez-Martinez H. 2003. Laboratory semen assessment and prediction of fertility: still utopia? *Reprod Domest Anim*, 38:312-318.

Rodriguez-Martinez H, Saravia F, Wallgren M, Roca J, Pena FJ. 2008. Influence of seminal plasma on the kinematics of boar spermatozoa during freezing. *Theriogenology*,70:1242-1250.

Saravia F, Wallgren M, Johannisson A, Calvete JJ, Sanz L, Pena FJ, Roca J, Rodríguez-Martínez H. 2009. Exposure to the seminal plasma of different portions of the boar ejaculate modulates the survival of spermatozoa cryopreserved in MiniFlatPacks. *Theriogenology*, 71:662-675.

Satake N, Elliott RM, Watson PF, Holt WV. 2006. Sperm selection and competition in pigs may be mediated by the differential motility activation and suppression of sperm subpopulations within the oviduct. *J Exp Biol*, 209:1560-1572.

Sousa AP, Amaral A, Baptista M, Tavares R, Caballero Campo P, Caballero Peregrín P, Freitas A, Paiva, Almeida Santos T, Ramalho Santos J. 2011. Not all sperm are equal; functional mitochondria characterize a subpopulation of human sperm with better fertilization potential. *Plos One*, 6:e18112

Thurston LM, Watson PF, Mileham AJ, Holt WV. 2001. Morphologically distinct sperm subpopulations defined by Fourier shape descriptors in fresh ejaculates correlate with variation in boar semen quality following cryopreservation. *J Androl*, 22:382-394.

van Dilla MA, Gledhill BL, Lake S, Dean PN, Gray JW, Kachel V, Barlogie B, Göhde W. 1977. Measurement of mammalian sperm deoxyribonucleic acid by flow cytoemetry. Problems and approaches. *J Histochem Cytochem*, 25:763-773.