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NEW FLOW CYTOMETRIC METHODS FOR EVALUATING BULL SEMEN[1]

Chelsey Kennedy[2] and Peter Sutovsky[2,3]

[2]Division of Animal Sciences
[3]Departments of Obstetrics, Gynecology and Women's Health
University of Missouri, Columbia, MO

Abstract

Bull fertility and semen quality are of paramount importance to the cattle industry. Fluorescent biomarkers measured by flow cytometry can aid in the detection of sub-fertile bulls as they can identify abnormal spermatozoa that may not be properly identified as inferior with standard methods of subjective semen analysis. This paper introduces the basics of flow cytometry, discusses fluorescent biomarkers of sperm quality in current use, and summarizes results from sperm proteomic studies in the bull.

Introduction

Sub-fertile bulls can cost producers a significant amount of money – they may contribute to delayed conception, prolonged calving season, reduced calf weaning weight, and increase the number of female culls. When used in a multiple-sire or low breeding pressure situation, a sub-fertile bull may be less evident, but in a single sire, high breeding pressure, or artificial insemination service, the fertility of the bull is of extreme importance (Kastelic and Thundathil, 2008). Breeding soundness evaluation of yearling bulls may give some indication of fertility, but conventional semen analysis is less than ideal – the results vary from one technician to another; it is time consuming; only a few hundred spermatozoa per sample are analyzed, and the agreement between microscopic assessments of sperm motility for different lab technicians can be low. The lack of precision in conventional semen analysis, coupled with the subjective nature of such an assessment, implies that some acceptable semen may be erroneously rejected, and at the same time semen of unacceptable quality may be used for inseminations (Christensen et al., 2005). A quick, precise, and accurate method for semen evaluation has been a goal of the breeding industry, and flow cytometry using fluorescent biomarkers may be the answer. Flow cytometry is fast, accurate, highly repeatable, and can analyze significantly more sperm per sample (up to 10,000) than standard semen analysis (Christensen et al., 2004). In addition to the speed, repeatability, and accuracy, flow cytometry allows close examination of numerous sperm characteristics, including sperm viability/membrane integrity (Evenson et al., 1982; Garner and Johnson, 1995; Garner et al., 1994), mitochondrial function and membrane potential (Evenson et al., 1982; Garner et al., 1997; Graham et al., 1990) chromatin structure (Bochenek et al., 2001;

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Evenson et al., 1980), and acrosomal status (Graham et al., 1990; Nagy et al., 2003; Thomas et al., 1997).

Flow Cytometry

Flow cytometry is a process in which fluorescently labeled cells (in this case, spermatozoa) travel individually at high speed (hundreds or thousands per second) through a flow cell, where they are illuminated by one or more lasers. This causes light scattering and fluorescence excitation of markers located on specific parts of the sperm, which is then picked up by photo-detectors and sent to a computer program. The computer program presents the information in the form of relative fluorescent intensity units, which are typically displayed as either scatter plots or histograms (Martinez-Pastor et al., 2010) Figure 1). The scatter plots and histograms can be analyzed and various sperm populations can be separated to produce information regarding fluorescence intensity, percentage of sperm population with certain fluorescent characteristics within a total sample, median fluorescence intensity, etc. One of the main concerns with analyzing spermatozoa by flow cytometry is the presence of non-sperm events in the sample such as immature forms of spermatogenic cells, bacteria, blood cells, tissue, and in the case of frozen-thawed semen, extender contaminants such as egg yolk particles. During the data analysis, these non-sperm events can be taken into account and most of the time can be eliminated from evaluation by gating of scatter diagram/histogram.

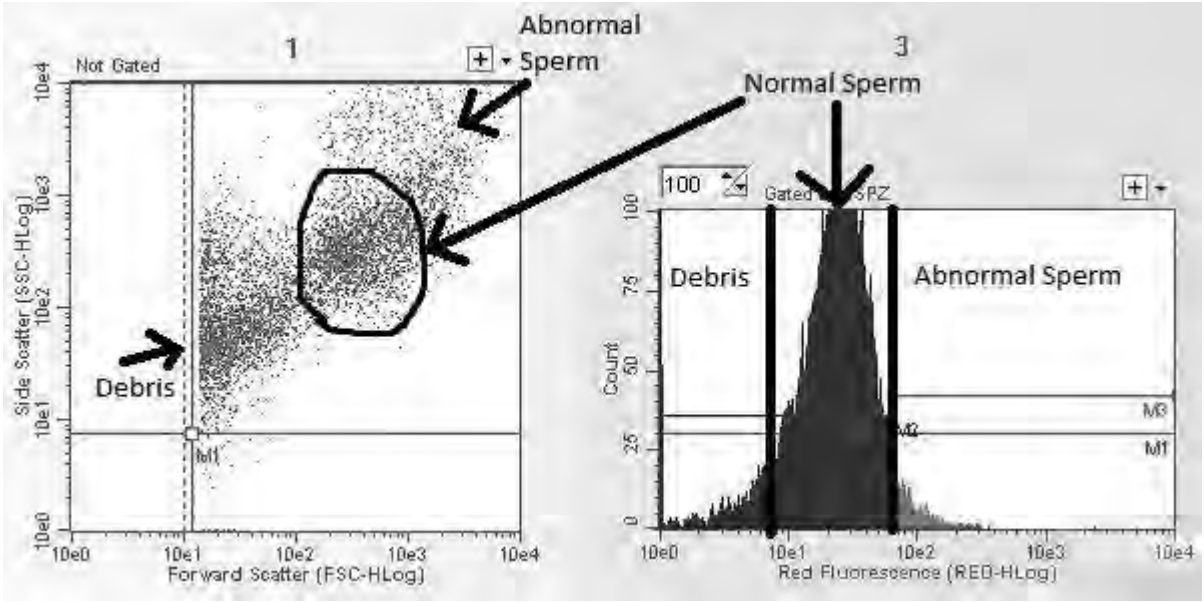

Figure 1: Typical scatter diagram of visible light (left) and a histogram of PAWP-induced fluorescence (right) yielded by flow cytometric measurement of a bull sperm sample. Normal spermatozoa are gated based on their size (forward and side scatter) in a scatter diagram, and based on their relative fluorescence in a histogram.

Vital Dye Based Sperm Flow Cytometry

When coupled with specific fluorochromes conjugated to biomarker or reporter molecules, flow cytometry can be used to analyze a variety of structural and functional characteristics of spermatozoa, including plasma membrane integrity, mitochondrial activity/mitochondrial membrane potential, acrosome integrity, chromatin structure, changes in the sperm surface induced by sperm capacitation, and certain forms of morphological abnormalities present in a sperm sample (Bochenek et al., 2001). Some of these biomarkers are still in their infancy but are showing great promise and are correlating well with the standard fertility parameters. Numerous biomarkers are available or in development for use in sperm evaluation with flow cytometry. Some, such as ubiquitin and PNA, are present only in poor quality sperm and are generally referred to as negative biomarkers. Others, such as those used for mitochondrial membrane potential (Evenson et al., 1982; Graham et al., 1990; Thomas et al., 1997), viability (Evenson et al., 1982; Garner et al., 1994; Thomas et al., 1997), and PAWP (Wu et al., 2007), are present in varying amounts depending on the quality of the spermatozoa.

The fluorescent probes, JC-1 and MitoTracker, are used for mitochondrial membrane potential track damage to the mitochondria that may occur after ejaculation or as a result of cryopreservation. The MitoTracker probe is taken up only by active mitochondria, and the reduced intensity of labeling in spermatozoa may also be reflective of malformations of the sperm tail midpiece/mitochondrial sheath. The JC-1 probe is transported into the interior of functioning mitochondria and senses the potential of the inner mitochondrial membrane. JC-1 emits green fluorescence when it exists as a monomer, but when the concentration of JC-1 inside the active mitochondria increases, the stain forms aggregates which fluoresce orange (Graham, 2001). Therefore, the mitochondria with high membrane potential fluoresce orange and those with medium to low membrane potential fluoresce green (Garner et al., 1997).

Depolarization of this membrane impairs the electron transport chain, the proton gradient and aerobic ATP production in the midpiece of the spermatozoa, thereby rendering its motility characteristics inadequate for fertilization (Nagy et al., 2003). A correlation between mitochondrial fluorescence intensity and sperm motility has been established (Garner et al., 1997).

Viability is another parameter that can be measured by flow cytometry, and is particularly useful since it has been found that sperm viability of both raw and frozen-thawed bull semen correlates well with non-return rates (Christensen et al., 2005). Currently, one of the most commonly used viability stain combinations is SYBR-14 and PI, sold commercially as LIVE/DEAD® Sperm Viability Kit. Both SYBR-14 and PI dyes target the same cellular component, DNA, eliminating the ambiguity that can arise when different components are targeted. With these biomarkers, the nuclei of live spermatozoa display green fluorescence because of integration of SYBR-14 and dead/dying cells with compromised membrane integrity stain orange because of passive PI-uptake through damaged plasma membrane. By combining the viability stains with other biomarkers additional sperm functions such as acrosomal integrity and mitochondrial function can be assessed(Gillan et al., 2005). To date, the SYBR-14/PI combination has been used to effectively identify live and dead sperm populations in bulls, boars, rams, rabbits, mice, rats, and

men, though it has also been used to stain spermatozoa of more exotic species such as tigers and chinchillas (Garner and Johnson, 1995).

Sperm capacitation includes a process of plasma membrane destabilization which may lead to physiological acrosome reaction upon sperm-egg binding, or to sperm cell death in absence of sperm-zona interaction, both of which are dependent on an influx of calcium ions in the sperm interior (Maxwell and Johnson, 1997). Therefore, the capacitation status of spermatozoa can be detected using the fluorescent antibiotic chlortetracycline (CTC), which traverses the sperm plasma membrane and enters intracellular compartments containing free calcium. Once inside, the CTC becomes negatively charged and binds to the calcium, increasing the CTC fluorescence (Maxwell and Johnson, 1997). This CTC-calcium complex proceeds to bind to hydrophobic regions of plasma/acrosomal membranes and produces distinct staining patterns based on capacitation status: capacitated (B-pattern), non-capacitated (F-pattern), and acrosome reacted (AR-pattern) (Saling and Storey, 1979). The CTC staining has been routinely used with fluorescence microscopy to visualize capacitation and AR in several species, including mouse (Saling and Storey, 1979), bull (Fraser et al., 1995), boar (Wang et al., 1995), stallion (Bergqvist et al., 2011), and men (Li et al., 2009) and has been adapted for use in flow cytometry by Maxwell and Johnson (Maxwell and Johnson, 1997).The collection of semen in livestock species is a less-than-sterile procedure. Despite rigorous attempts at cleanliness, bacterial contamination from the penis and prepuce, collection equipment, and handlers is possible (Yaniz et al., 2010). Consequently, bacteria may compromise semen quality and contaminate the receiving female's reproductive tract. Though more frequently studied in humans, a variety of bacteria have been identified via culture of semen samples, and certain bacteria have been shown to have detrimental effects on semen quality in several domestic species (Akhter et al., 2008; Althouse et al., 2000; Aurich and Spergser, 2007). Fluorescent markers such as SYBR-Green 1 have been adapted to identify bacteria in semen, and the use of flow cytometry for bacterial counts is becoming more routine (Tripp, 2008). This enables the producer to inspect bacterial counts in collected semen prior to cryopreservation and use an extender containing antibiotics, if necessary (Yaniz et al., 2010).

The sperm chromatin structure assay (SCSA) detects abnormal chromatin structure consisting of abnormal nuclear proteins (%HDS) and sperm DNA fragmentation index (%DFI). The DNA fragmentation index is the proportion of sperm containing fragmented DNA and is calculated from a histogram obtained from the ratio between red and total (red+green) fluorescence. High DNA stainability (% HDS) is calculated based on the percentage of spermatozoa with high levels of green fluorescence, representing immature spermatozoa with incomplete chromatin decondensation (Giwercman et al., 2010). In a study by Bochenek et al. (2001) the sperm chromatin structure assay was performed on mature bulls considered qualified for AI. The results showed that ejaculates positively evaluated at the AI stud via microscope contained as much as 23.8% chromatin-defective spermatozoa (Bochenek et al., 2001). Furthermore, SCSA values were shown to correlate with fertility parameters and the percentage of spermatozoa with chromatin defects varied over several weeks, suggesting that defective chromatin structure could be a variable trait that can be affected by disturbances in the spermatogenetic process or external factors such as semen extender, heat stress, or chemicals (Bochenek et al., 2001).

Lectins

The visualization of the acrosomal status could be an important factor to consider when evaluating semen quality, as some instances of male infertility could be the result of a lack of sperm with functional intact acrosomes at the time of ejaculation (Odhiambo et al., 2011). Acrosomal integrity of spermatozoa can be measured by using fluorescently labeled plant lectins, proteins that recognize and bind glucosidic residues in different parts of the acrosomal membrane. *Pisum sativum* agglutinin (PSA) derived from the pea plant and *Arachis hypogaea* agglutinin (PNA) derived from the peanut plant are the most commonly used because of their specificity (Graham, 2001). However, PSA has a tendency to bind to the egg yolk particles in the extenders and has slightly less specific binding, so in agreement with other scientists (Gillan et al., 2005; Graham, 2001), the authors' preference is to use peanut agglutinin (PNA). PNA shows a high affinity and strong specificity for disaccharides with terminal galactose, especially the D Gal α (1,3) D GalNAc disaccharide, and binds to the outer acrosomal membrane, which becomes exposed during the acrosome reaction. Spermatozoa with reacted, damaged, or abnormally formed acrosomes acquire green fluorescence after PNA labeling, while intact, normal acrosomes have no fluorescence (Nagy et al., 2003). We have found that PNA values have shown correlations with the conventionally established parameters of sperm morphology and sperm concentration, as well as with several industry-provided indexes reflective of bulls' reproductive performance in AI services (unpublished data/in preparation). A study by Thomas et al (1997) compared acrosomal integrity as determined by PNA via flow cytometry to the standard microscopic morphology assessments and found that the percentage of spermatozoa with normal acrosomes, relative to the values obtained using fluorometric methods, appeared to be understated in bulls producing semen of low quality and overstated in bulls producing semen of high quality, further illustrating the subjective nature of microscopic semen analysis and the tendency to bias visual examinations (Thomas et al., 1997). A third lectin, LCA from the lentil plant (*Lens Culinaris*) has also been used to evaluate bull sperm quality. LCA shows a strong specificity to D-glucose and D-mannose residues and binds to the entire surface of defective spermatozoa, but only to the acrosomal surface in normal spermatozoa. Consequently, distinct histograms of LCA-induced fluorescence are seen in normal vs. defective spermatozoa. In our yet to be reported data, we have found a positive correlation between LCA and ubiquitin staining and a negative correlation of LCA with % normal sperm morphology. Thus, it appears that LCA can be useful in the detection of abnormal spermatozoa via flow cytometry (unpublished data).

Protein Biomarkers of Sperm Quality

Potential sperm quality/fertility biomarkers include proteins that are exclusively associated with certain types of sperm defects ("negative" fertility markers) and proteins more abundant in morphologically and functionally normal spermatozoa ("positive markers"). One of the protein biomarkers of bull sperm quality that have been studied in depth is ubiquitin. Abnormal spermatozoa are tagged by ubiquitination of the plasma membrane/sperm surface during epididymal passage (Baska et al., 2008). Though some of these ubiquitin tagged spermatozoa may disintegrate and be removed in the epididymis, many abnormal spermatozoa appear in the ejaculate, and their increased content is indicative of poor semen quality, or even infertility (Sutovsky et al., 2003). Increased binding of fluorescently-labeled anti-ubiquitin antibodies to the sperm surface reflects the occurrence of sperm abnormalities, which is then detected by the

flow cytometer as an increase in the relative fluorescence induced by the presence of ubiquitin on the sperm surface. Ubiquitin as a sperm biomarker has been assessed in numerous species including men (Sutovsky et al., 2001), stallions (Sutovsky et al., 2003), bulls (Sutovsky et al., 2002), and boars (Kuster et al., 2004), and has been found to correlate with infertility and indications of poor sperm quality, including primary and total morphological defects (Purdy, 2008). Though not yet published, our data indicate that ubiquitin may be correlated with non-return rate as well. In a human fertility trial, it was found that higher levels of ubiquitin in the sperm sample also correlated negatively with the percentage of cleaved embryos and the percentage of embryos with two pronuclei after infertility therapy by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (Ozanon et al., 2005). If the decreased embryo cleavage and pronuclei-formation rates also apply to cattle AI, it can be inferred that early embryo loss could account for an increased non-return rate.

Ubiquitin has also been used for validation of other candidate biomarkers of sperm quality, further illustrating the utility of multiple stains during flow cytometric analysis. A study by Sutovsky et al. (2007) examined the correlation between semen platelet activating factor-receptor (PAFr) and ubiquitin labeling, with interesting results (Sutovsky et al., 2007). The platelet activating factor (PAF) is an important phospholipid mediator in reproduction and, its sperm plasma membrane receptor PAFr, has a positive association with sperm motility and high fertility history in boars (Roudebush and Diehl, 2001). Sutovsky et al. (2007) examined the relationship between ubiquitin and PAFr via flow cytometry during breeding soundness evaluations in yearling bulls. Contrary to the association shown in swine, increased PAFr content was indicative of increased white blood cells (WBC) in bull semen samples, and PAFr-induced fluorescence correlated negatively with several BSE parameters (palpation, scrotal circumference, and satisfaction of evaluation). A positive correlation was found at the same time with semen ubiquitin content (Sutovsky et al., 2007). Without the additional parameter of ubiquitin expression, an increased PAFr expression could have resulted in an incorrect assumption of high semen quality.

One biomarker that correlates negatively with high levels of ubiquitin is PAWP, the post-acrosomal, ww-domain binding protein. PAWP is a novel protein found only in the post-acrosomal sheath (PAS) of the spermatid, within which it resides in the protective capsule enveloping the sperm nucleus, the perinuclear theca. PAWP first appears during spermatid elongation, coinciding with the time frame in which spermatids acquire their egg-activating ability (Wu 2007). Though the downstream elements of PAWP-dependent oocyte activation cascade are currently under investigation, PAWP triggers meiotic resumption and pronuclear development. A proper integration of PAWP in the sperm PAS is thought to be reflective of bulls' sperm quality/fertility. When flow cytometry is performed on anti-PAWP antibody labeled spermatozoa, a threshold of acceptable PAWP content can be established. Anything above or below the threshold amount and a spermatozoa would be considered abnormal. In normal spermatozoa, PAWP fluorescence formed a regular band around the proximal PAS. Defective spermatozoa displayed various anomalies of PAWP labeling including a jagged or abnormally wide band, an irregular spot or completely absent labeling(Sutovsky and Oko, 2011). Depending on which population is analyzed, PAWP can have a positive or a negative correlation with secondary sperm morphology, conception rate, number of services, non-return rate and

residual value reflective of bulls' performance in AI service, and has also been found to have a negative correlation with the ubiquitin biomarker (Figure 2 and unpublished data/in preparation).

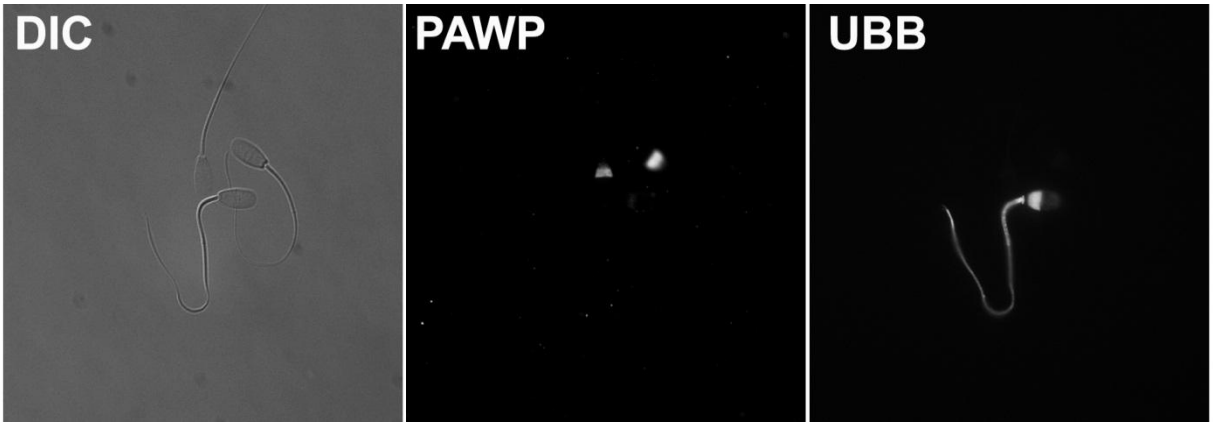

Figure 2. Dual immunofluorescence labeling of PAWP and ubiquitin (UBB) in bull spermatozoa. A spermatozoon displaying UBB labeling lacks PAWP on its postacrosomal sheath. Parfocal transmitted light image was acquired by using differential interference contrast (DIC) optics.

Heparin binding proteins (HBP) secreted by the seminal vesicles, prostate, and bulbourethral glands are present in the seminal fluid and bind to spermatozoa after ejaculation. The presence of a specific heparin binding protein, HBP-30, on the sperm plasma membrane has been correlated with increased fertility in bulls, inspiring a synonym the fertility-associated antigen (FAA; Bellin et al., 1998). High semen content of FAA was predictive of high fertility in bulls of several breeds in trials grouping bulls based on the presence or absence of FAA (Bellin et al., 1994; Bellin et al., 1996; Bellin et al., 1998). Despite the fluctuation of absolute fertility values, groups of FAA-positive bulls were consistently more fertile (by 9 to 40 percentage points) than groups of FAA-negative bulls (Bellin et al., 1998). Furthermore, cows covered by FAA positive bulls were impregnated earlier in the breeding season, resulting in increased numbers of older and heavier calves at weaning (Bellin et al., 1998). Based on this information, an artificial insemination trial on beef cows inseminated using semen from FAA positive or FAA negative bulls was performed (Sprott et al., 2000). The results of this insemination trial support the usefulness of the fertility associated antigen as a biomarker of sperm quality: Pregnancy rates in females inseminated with FAA-negative bull semen ranged from 2.8-69.6%, while females inseminated with FAA-positive bull semen had a pregnancy rate of 22.1-91.3% (Sprott et al., 2000). The presence or absence of FAA can only be determined via biochemical analysis—it has no relation to breeding soundness or serving capacity (Sprott et al., 2000).

Clusterin, an acidic glycoprotein produced in the testis and epididymis, has been associated with sperm quality in several species, including ram (Tung and Fritz, 1985), rat (Sylvester et al., 1991; Sylvester et al., 1984), bull (Ibrahim et al., 1999), and men (O'Bryan et al., 1990). In human semen, clusterin was detected only on abnormal spermatozoa (O'Bryan et al., 1990), and the bull and ram spermatozoa that exhibited morphological defects strongly reacted with anti-clusterin antibody (Ibrahim et al., 1999). Clusterin is associated with cell damage in several disease conditions (Ibrahim et al., 2000) but the reason for clusterin accumulation in abnormal

spermatozoa is unknown. The result of a scrotal insulation trial in rams (Ibrahim et al., 1999) suggests that the accumulation of clusterin on abnormal spermatozoa may indicate unfavorable testicular conditions or individual germ cell aberrations (Ibrahim et al., 2000). The incidence of clusterin-positive bull spermatozoa, as determined via flow cytometry, negatively correlates with non-return rate and estimated relative conception rate (Ibrahim et al., 2000). In addition, an inverse relationship between clusterin-positive bull spermatozoa and pre/post-thaw motility has also been established, suggesting that clusterin is another potential biomarker of bull fertility.

Bull Sperm Proteomics: Searching for New Biomarkers

In the United States, roughly 70% of dairy cows are bred via artificial insemination (Killian, 1999). Of that 70%, only about 50% of inseminations result in a full term pregnancy, in part as a result of a lack of thorough understanding of the molecular events and mechanisms that determine the fertilizing potential of a semen sample (Peddinti et al., 2008). In order to develop new biomarkers to assess the quality of a semen sample, we must first develop an understanding of these mechanisms through proteomics. Proteomics refers to the qualitative and quantitative comparisons of proteomes to identify cellular mechanisms which are involved in biological processes. However, proteomics is not only about the identification of such mechanisms; it also involves the study of protein structure, localization, posttranslational modifications, protein-protein interactions, biological activities, and function (Strzezek et al., 2005). Sperm proteomics can be used to discover new biomarkers of fertility, such as those described above. The proteomics of seminal plasma and epididymal fluid could be arguably just as important as sperm proteomics because spermatozoa acquire numerous sperm surface proteins that convey fertilization potential in the epididymis (Sutovsky, 2009). Comparison of bull sperm proteomes between fertile and sub/infertile bulls (Peddinti et al., 2008) as well as proteomic characterization of bovine seminal plasma (Kelly et al., 2006; Moura et al., 2007) have given some insight into which proteins at what levels are indicative of fertility or infertility.

New Instrumentation

As new technology is developed, instrumentation is updated or created to make use of the new biomarker-based methodology. The development of biomarkers is no exception, and new state of the art flow cytometers, including dedicated sperm flow cytometers have been introduced recently. These include the ImageStream high speed quantitative imaging cytometer (Amnis Corp., Seattle, WA) and the EasyCyte Plus 'bench-top' micro-capillary flow cytometer(IMV Technologies, L'Aigle, France).

In a study performed by Odhiambo et al (2011), the EasyCyte Plus flow cytometer was used to validate a dual ubiquitin-PNA based bull sperm assay. The EasyCyte Plus proved easy to use and can be operated by anyone who has completed the IMV Technologies web-based training program, which takes less than four hours—no expertise is needed. Setup is fast and the instrument can be fully operational in less than 15 minutes (Odhiambo et al., 2011). In addition to the quick setup, EasyCytePlus has an automated self-cleaning cycle without the large-volume fluidics found in conventional flow cytometers. When the results of the ubiquitin-PNA trial were compared between the EasyCyte Plus and the conventional flow cytometer (Becton Dickinson FACScan), both instruments produced robust evaluations of the semen samples

(Odhiambo et al., 2011). The relative affordability, compact 'bench-top' design, dedicated sperm analysis software and reliable results make the EasyCyte Plus an attractive choice for andrologists.

The ImageStream high speed quantitative imaging cytometer (Amnis Corp., Seattle, WA) was evaluated in another sperm biomarker trial by Buckman et al. (2009). The ImageStream combines the standard fluorescence intensity measurements with bright field and epifluorescence imaging, enabling it to directly correlate biomarker intensity with individual cell morphology, something standard flow cytometers cannot accomplish (Tai). This instrument captures up to six different images (four channels of fluorescence, side scatter, and bright field) of each passing cell at approx. 100 cells/second with a resolution comparable to that of a 40x microscope-lens magnification. It is equipped with image analysis tools that can measure parameters related to fluorescence intensity, cell size, shape, texture, and localization (Buckman et al., 2009). In addition, the ImageStream results correlate well with conventional flow cytometry results. However, the cost of this instrument makes it more suitable for core facilities shared by basic researchers rather than for andrology laboratories.

Concluding Remarks

The advent of sperm quality biomarkers and the implementation of flow cytometry should benefit the cattle industry greatly. Being able to identify markers of good fertility as well as poor fertility in a semen sample in a fast and objective manner could reduce the need for multiple inseminations and prevent expenses covering offspring testing of sub-fertile producing poor pregnancy rates in AI service. While the idea of using flow cytometry is new and some of the biomarkers are still being developed, an increase in speed, accuracy, and precision in the assessment of fertility in bulls should be welcomed by the cattle industry. Even if flow cytometry were only used to determine sperm viability and concentration, it could potentially still lead to more straws of semen being produced, and better quality control overall, which leads to an increase in profits. Finding the sub-fertile bulls that "fly under the radar" could potentially save producers considerable amounts of money, especially if sub-fertility could be determined early on before the bulls reach breeding age. Furthermore, flow cytometry could be used to aid in the development of new cryopreservation techniques, as some scientists are testing the effect of cryopreservation on various sperm characteristics, such as organelle function and viability (Thomas, 1998), acrosomal integrity and viability (Thomas, 1997), and mitochondrial function and viability (Thomas, 1997).

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