Sex-Sorting Sperm Using Flow Cytometry/Cell Sorting

Article *in* Methods in molecular biology (Clifton, N.J.) · August 2013

DOI: 10.1007/978-1-62703-038-0_26 · Source: PubMed

3 authors, including:

George Seidel
Colorado State University
189 PUBLICATIONS **4,727** CITATIONS

SEE PROFILE

Chapter 26

Sex-Sorting Sperm Using Flow Cytometry/Cell Sorting

Duane L. Garner, K. Michael Evans, and George E. Seidel

Abstract

The sex of mammalian offspring can be predetermined by flow sorting relatively pure living populations of X- and Y-chromosome-bearing sperm. This method is based on precise staining of the DNA of sperm with the nucleic acid-specific fluorophore, Hoechst 33342, to differentiate between the subpopulations of X- and Y-sperm. The fluorescently stained sperm are then sex-sorted using a specialized high speed sorter, MoFlo® SX XDP, and collected into biologically supportive media prior to reconcentration and cryopreservation in numbers adequate for use with artificial insemination for some species or for in vitro fertilization. Sperm sorting can provide subpopulations of X- or Y-bearing bovine sperm at rates in the 8,000 sperm/s range while maintaining; a purity of 90% such that it has been applied to cattle on a commercial basis. The sex of offspring has been predetermined in a wide variety of mammalian species including cattle, swine, horses, sheep, goats, dogs, cats, deer, elk, dolphins, water buffalo as well as in humans using flow cytometric sorting of X- and Y-sperm.

Key words: Sperm sorting, Flow cytometry, Predetermination of sex, X- and Y-sperm, Mammals, Hoechst 33342, DNA content

1. Introduction

Mammalian sperm can be effectively separated into relatively pure living populations of X- and Y-chromosome-bearing sperm using flow cytometry/cell sorting. In addition to describing procedures, this review provides the background on how conceptual advances originating from genetics, applied gamete biology, biophysics, and computer science were integrated in development of a commercial method to predetermine the sex of mammalian offspring.

1.1. Biological Basis

Examination of bovine chromosomes indicated that a total chromosome length difference of 4.2% existed between karyotypes of bulls and cows due to X-and Y-chromosome differences (1). This finding suggested a potential size difference between a sperm carrying the X-chromosome compared to one carrying a

Douglas T. Carrell and Kenneth I. Aston (eds.), *Spermiogenesis and Spermatogenesis: Methods and Protocols*, Methods in Molecular Biology, vol. 927, DOI 10.1007/978-1-62703-038-0_26,

Y-chromosome. It also had been reported that a potential difference in dry mass exists between sperm heads of X- and Y-chromosome-bearing sperm (2). Thus, a difference in DNA content between sperm bearing the X-chromosome and those bearing the Y-chromosome existed. However, measurement of the DNA content of individual sperm proved to be difficult due to the asymmetry of mammalian sperm head shape (3, 4).

1.2. Precise Measurement of Sperm DNA Content

The initial indication that differences in DNA content of mammalian sperm could be determined utilized coaxial flow cytometry and revealed two fluorescent populations of demembranated nuclei of X- and Y-human sperm (5, 6). This was accomplished using the first fluorescence-based commercial analytical flow cytometer developed by Wolfgang Göhde in 1968–1969 and marketed as the Phywe ICP 11(7) (German patent application DE1815352). The DNA content of asymmetric cells also could be measured precisely in an orthogonal flow cytometer if the cells were hydrodynamically orientated so that fluorescence could be accurately measured from the flat surface of the nucleus (8). Pinkel et al. (9) demonstrated that such an orienting system could readily resolve two fluorescent populations of sperm stained with a nucleic acid specific dye 4′-6-diamidino-2-phenylindole (DAPI) to reflect DNA content differences. The presumed sex-chromosomal origin of these fluorescence differences was supported by measurements of sperm from mice possessing the Cattanach translocation, where a piece of chromosome 7 had been translocated to the X-chromosome (10). Flow cytometric examination of the DNA content of sperm from Cattanach mice showed a 5.1% difference in the two sperm populations compared to the 3.3% for sperm from normal mice. Quantitative differences in the DNA content of the X- and Y-chromosome-bearing sperm of several heterogametic mammals including cattle (*Bos taurus* and *Bos indicus*), sheep (*Ovis aries*), swine (*Sus scrofa*), and rabbits (*Oryctolagus cuniculus*) were demonstrated using the homogametic Z sperm of roosters (*Gallus domesticus*) as a standard (11). The above described results strongly suggested that the two peaks demonstrated in mammalian sperm represent the X- and Y-chromosome-bearing sperm. This flow cytometric approach identified two populations of sperm with characteristic suggesting that they were the X- and Y-chromosome-bearing populations, but this coaxial analytical system could not physically sort cells.

1.3. Developments in Flow Cytometry

The development of flow cytometry began in the 1960s when Louis Kamentsky and associates first measured ultraviolet absorption in cancer cells using a fluidic system (12, 13). Subsequent work at the Los Alamos National Laboratory (8) and Stanford University (14) resulted in development of a flow sorting system called a fluorescence-activated cell sorter (FACS) that used fluorescence rather than absorption as a measurement criterion.

The first actual sorting of mammalian sperm into separate populations was achieved with sperm nuclei of the vole, *Microtus oregoni* (15). The DNA content difference of the sex determining Y- and O-sperm from this rather unique species is 9% because half of the sperm contain a Y-chromosome, while the other half of the sperm have no sex chromosome. The X-chromosome is reconstituted at fertilization in this species (15). Although not very efficient, sort purities of 82–95% were achieved with nuclei of the "O" sperm and 72–83% for the Y fraction. Thus, the ability to separate the nuclei of sperm of mammals into two populations relative to DNA content was established using flow cytometric sorting.

1.4. DNA Content Measurement in Living Sperm

The demonstrated DNA content difference between the two populations of mammalian sperm is large enough that the possibility of sorting the sperm into separate living populations was undertaken. Sperm sorting technology was established at the USDA Beltsville Agricultural Research Center (BARC) by purchase and modification of a Coulter EPICS V® Flow Cytometer/Cell Sorter (Beckman Coulter, Hialeah, FL and Fullerton, CA, USA) with the intent of separating the X- and Y-sperm of domestic livestock. At that time the membrane impermeant fluorescent staining system that was used to measure sperm for DNA content required removal of the cell membranes, thereby killing sperm (11, 15). Initial efforts demonstrated that dead X- and Y-sperm populations could be produced with purities greater than 85–90% for each population (16). It wasn't until Johnson et al. (17) altered the staining process that living sperm could be sorted according to their DNA content. The membrane permeant bisbenzimidazole fluorescent dye, Hoechst 33342, readily differentiated between the two populations of living sperm according to their DNA content (18). This initial advancement in staining with Hoechst 33342 was followed by development of the ability to sort living sperm into populations of the purportedly viable X- and Y-sperm populations at 85–90% purity (see Fig. 1) (17, 18). The efficacy of this sperm sorting technology was tested using surgical insemination of the two sorted sperm populations into the oviducts of rabbits (17). In rabbits, the resultant offspring from inseminations with the purported X-sperm populations were 94% female, while those females inseminated with the purported Y-sperm population produced 81% males (17). Similar data were obtained when this approach was applied to the domestic swine (19). This successful production of offspring with significantly skewed sex ratios in rabbits and swine provided the basis of a patent (US Patent #692958, 04/26/1991). The USDA then licensed the technology to Animal Biotechnology, Cambridge, Ltd, which was later to become Mastercalf Ltd, Cambridge, UK, for field testing. In this first field testing of the sorting technology, a Facstar Plus® (BD Biosciences, San Jose, CA, USA) was modified to sort living bovine sperm. This initial project utilized in vitro

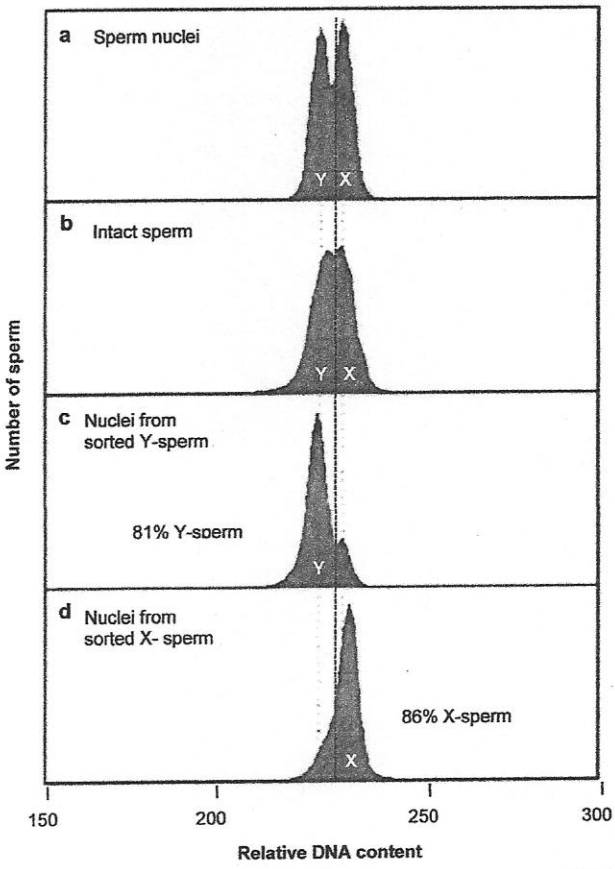

Fig. 1. Illustration showing a comparison of the relative DNA content of Hoechst 33342-stained rabbit sperm nuclei, living X- and Y-sperm, nuclei recovered from Y-sorted sperm and X-sorted sperm. (**a**) Example illustration of the 3.0% DNA content difference between X- and Y-sperm nuclei; (**b**) Example of the relative DNA content difference of intact living sperm stained with Hoechst 33342; (**c**) Representation of the frequency distribution of DNA content from reanalysis of rabbit sperm nuclei prepared from an aliquot of Y-sorted living sperm; and (**d**) Representation of the frequency distribution of DNA content from reanalysis of rabbit sperm nuclei prepared from an aliquot of X-sorted living sperm (Adapted from Johnson et al., 1989 (17)).

fertilization (IVF) with sex-sorted bovine Y-sperm in an effort to produce male embryos. The resultant embryos were then cryopreserved and later transferred to recipients on 12 Scottish farms (20, 21). From 106 twin transfers of the bovine embryos that were fertilized by Y-sorted sperm to recipient cows, 37 male and 4 female calves were born. Thus, 90% were male as predicted from the Y-sperm DNA content observed during the sex-sorting process. The sorting process at that time, about 600 sperm/s, was not fast enough to produce adequate numbers of sperm for the use of sex-sorted sperm with artificial insemination (AI).

The membrane impermeant DNA-specific dye, propidium iodide (PI), was added during the staining process to identify dead and injured sperm (22). The PI quenches the Hoechst 33342

stain, thereby providing a means of selectively eliminating these damaged cells from the sorted product by selective gating. This staining process was further improved by substituting the food dye, FD&C #40 (Warner Jenkinson, St. Louis, MO, USA) for the PI, which is a potential mutagen (23, 24).

Development of a high speed sorter at Lawrence Livermore National Laboratory (25) and significant improvements in data acquisition speed (26) increased the speed at which sperm could be sorted. This high speed sorting system was commercialized by Cytomation, Inc. (Now part of Beckman Coulter, Inc, Miami, FL, USA) as the MoFlo®. This sperm sorting system was further enhanced by development of a novel nozzle-tip that oriented approximately 70% of the sperm in the laser beam as they passed through the sorter compared to 30% orientation capable with the original sperm sorter (27, 28). An original MoFlo instrument was installed at the BARC and modified for sperm sorting by installing a cell orienting nozzle-tip, making it capable of measuring and handling analysis rates close to 15,000 sperm/s (Rens et al., US Patent 5,985,216). The instrument, which had been modified specifically for sperm sorting, is called a MoFlo® SX.

2. Flow Cytometric Sorting of X- and Y-Sperm

2.1. Sperm Sample Preparation

Semen is collected and prepared for sorting using carefully cleansed equipment and sterilized media. The semen sample is examined for volume, and antibiotics in 20 μL/mL of semen (Tylosin (100 μg/mL), Gentamicin (500 μg/mL), and Linco-Spectin (300/600 μg/mL; final concentrations) are added to the raw semen prior to determinations of sperm concentration and motility. The raw semen is then stored up to 7 h with aliquots diluted approximately hourly to a sperm concentration of 200×10^6/mL with a modified Tyrode's balanced salt solution (staining TALP) consisting of 95.0 mM NaCl, 3.0 mM KCl, 0.3 mM NaH_2PO_4, 10.0 mM $NaHCO_3$, 0.4 mM $MgCl{\cdot}6H_2O$, 2.0 mM pyruvic acid, 5.0 mM glucose, 25 mM Na lactate, 40.0 mM HEPES, 3 mg/mL BSA, and 30 μg/mL gentamycin sulfate (24).

2.2. Sperm Staining

An aliquot of the bull semen which had been diluted to 200×10^6 sperm/mL in staining TALP is then stained with 50.4 μM Hoechst 33342 (9.0 μL Hoechst 33342/mL, from a 5 mg/mL distilled H_2O stock solution of Hoechst 33342) and placed in a 34°C water bath for incubation for 45 min. After removal from the water bath, 100 μL/mL of 20% egg yolk in a TALP is added, which makes the final egg yolk concentration, 2%. The stained sperm samples are then filtered using 50 μm sterile nylon mesh filters (CellTrics®, Partec, Münster, Germany) to remove clumped

sperm, media aggregates and seminal debris. Identification of nonviable sperm is accomplished by adding 2 μL/mL of a 1% FD&C #40 food coloring in TALP (23) to quench Hoechst staining of the DNA in membrane-damaged sperm.

2.3. Sorting Equipment

After stoichiometrically staining the sperm with Hoechst 33342, they are pumped in a stream in front of a UV laser beam (wavelength ~355 nm) to excite the Hoechst 33342-stained DNA to differentiate between the X- and Y-chromosome-bearing sperm. Most stained sperm are oriented as they pass in front of the laser so that the flat surface of the sperm nucleus can be used to precisely measure the DNA content (see Fig. 2). This orientation of the perm nucleus is accomplished using the hydrodynamic forces of

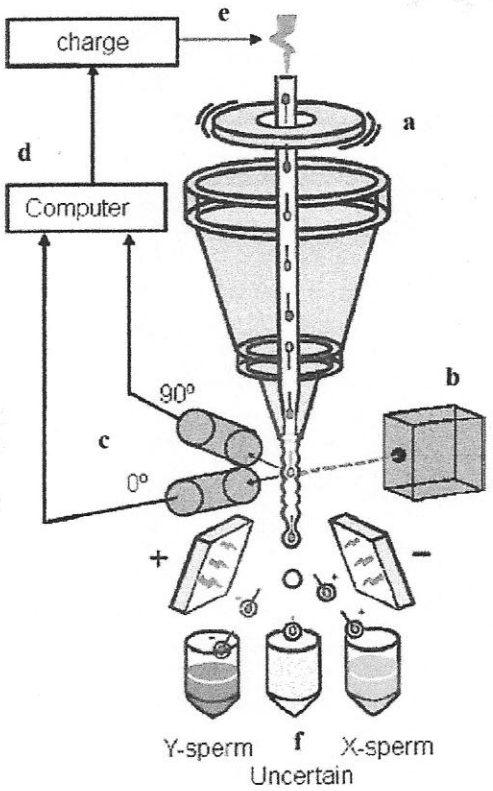

Fig. 2. Flow cytometer/sperm sorter system showing how Hoechst 33342-stained sperm are pumped through the sorting system including, (**a**) the piezoelectric crystal vibrator (~70,000/s) that causes the stream to form droplets as it exits the system; (**b**) the pulsed UV laser that illuminates the sperm as they flow through the system in the stream; (**c**) the two fluorescence photodetectors that capture the fluorescence emissions at 0° and 90°; (**d**) the fluorescence signal is quantified and categorized as X, Y, or uncertain; (**e**) positive, negative, or no charge is applied to the droplets as they emerge from the stream; (**f**) as the charged droplets pass between two oppositely charged plates they are deflected into either the X- or Y-catch tubes according to their DNA content while droplets without sperm, those with compromised cell membranes, or those with sperm of uncertain DNA content pass directly into the unsorted container. Adapted from Garner, 2001 (46).

the pressurized sheath fluid that surrounds the sample stream as it is pumped into the nozzle of the flow sorter. The sheath fluid used for this purpose is a TRIS-based buffer consisting of TRIS (hydroxymethyl) aminomethane (tris, 197.0 mM), citric acid monohydrate (55.4 mM), and fructose (4.75 mM) (24). The emitted fluorescence (~460 nm) of the oriented sperm is digitally quantified by dual orthogonal photodetectors which are situated at 0° and 90° to the laser beam to optimize capture of the fluorescence and to determine if a sperm is properly oriented for precise measurement of the DNA content using the 0° detector. This system can provide sorted subpopulations of X- or Y-bearing sperm at rate of 8,000 sperm/s at a purity of 90%. As the sperm exit the orienting nozzle, the stream is broken into individual droplets by a piezoelectric crystal vibrator. Either a negative or positive charge is applied to droplets containing sperm relative to their DNA content. The charged droplets then pass in front of two charged plates, one positive and one negative, such that they are deflected into one of three collection tubes, X-bearing sperm, Y-bearing sperm, and one for undetermined DNA content, membrane-damaged sperm with quenched fluorescence or no sperm (see Fig. 2).

The current state-of-the-art sperm sorter is a MoFlo® SX XDP, which uses digital technology to overcome problems associated with the coincidence when two sperm fall within the processing time window of the sorting electronics while exiting the nozzle at nearly the same time. Previous versions of the MoFlo® SX instrument were not able to differentiate between two closely positioned sperm due to processing speeds of the electronics and overlap of their fluorescence emissions. The configuration of a dual headed MoFlo® SX XDP includes two sort chambers supported by one laser (see Fig. 3).

2.4. Instrument Configuration

The excitation and fluorescence collection scheme for sorting sperm according to their DNA content requires that the stained sperm nucleus be oriented with its flat surface perpendicular to the 0° detector (see Fig. 4a). The 90° detector is utilized simultaneously to determine if that particular sperm is properly oriented. When the edge, rather than the flat surface, of the sperm head is oriented towards a detector this produces an approximate 2:1 relative fluorescence compared to that which is measured by the flat surface (see Fig. 4b). This is due to a lensing effect of the asymmetric sperm head. The dead sperm along with those with damaged membranes are identified by the uptake of the red food dye, which quenches the emission of the Hoechst 33342, thereby resulting in less fluorescence emission (see Fig. 4b).

2.5. Setting Sort Gates

The sperm populations identified by Hoechst 33342 staining, X- and Y- and dead sperm, are gated to achieve the desired purity of the population to be sorted and to identify damaged and dead

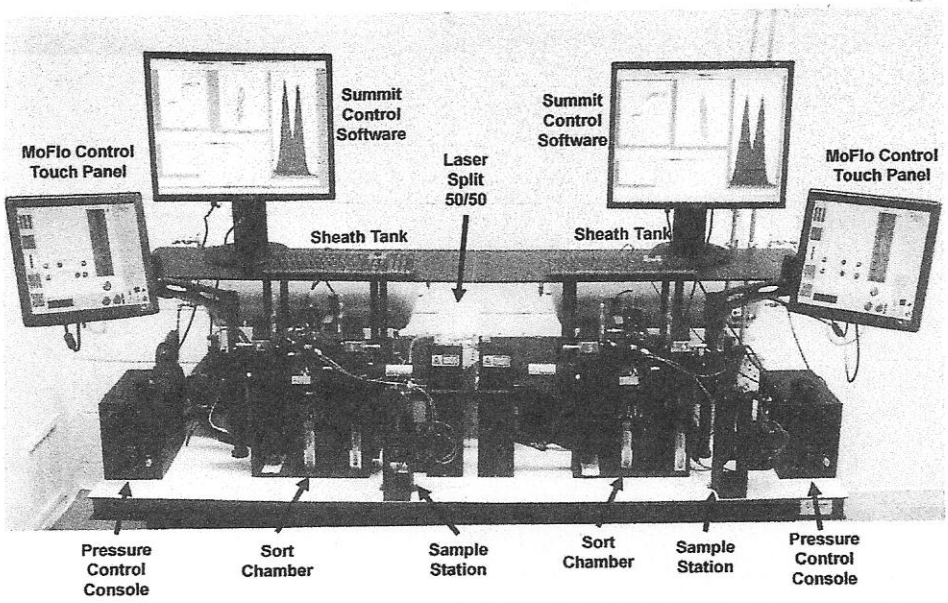

Fig. 3. The configuration of a dual-headed flow cytometer/sperm sorter, the MoFlo® SX XDP (Beckman Coulter, Inc., Miami, FL, USA), is shown illustrating the use of one pulsed laser with two sorting chambers. The various components; pulsed NdYag: Quazi-CW Mode Tripled laser, pressurized sheath tanks, sample stations, sort chambers, pressure control consoles, MoFlo® control touch panels, and the Summit® control software monitors are shown.

sperm (see Fig. 5). Once the desired population has been identified through bivariate analyses, it can be secondarily gated and magnified using the 0° detector to enhance the purity and recovery of the sorted population (see Fig. 5).

2.6. Sperm Sorting Procedure

Once the sort gates are set, the selected populations are sorted into a 15 or 50 mL conical centrifuge tube containing approximately 4 mL of 4% egg yolk in a TALP medium. The actual volume of EYC medium in this "catch tube" may vary based on the number of sperm to be sorted. Approximately 8,000 sperm/s of one sex can be sorted with the MoFlo®SX. Thus, approximately 28×10^6 sperm can be sorted during a 60 min sorting session using this equipment. The sort speeds are somewhat slower if both X- and Y-sperm populations are sorted. The catch tubes should be swirled gently every few minutes, after about 500,000 sperm have been sorted, to mix the sorted sperm with the egg yolk-containing medium in the catch tube to minimize oxidative damage to the sperm membranes.

2.7. Reconcentration of Sperm

The sorting process results in considerable dilution of the sperm, which necessitates centrifugation of the sperm to achieve a sperm concentration suitable for subsequent packaging and utilization in AI or IVF. The sperm that had been sorted into the medium in the catch tube are then slowly cooled to 5°C to prevent cold shock by placing the catch tube containing the prepared sperm in a 600 mL

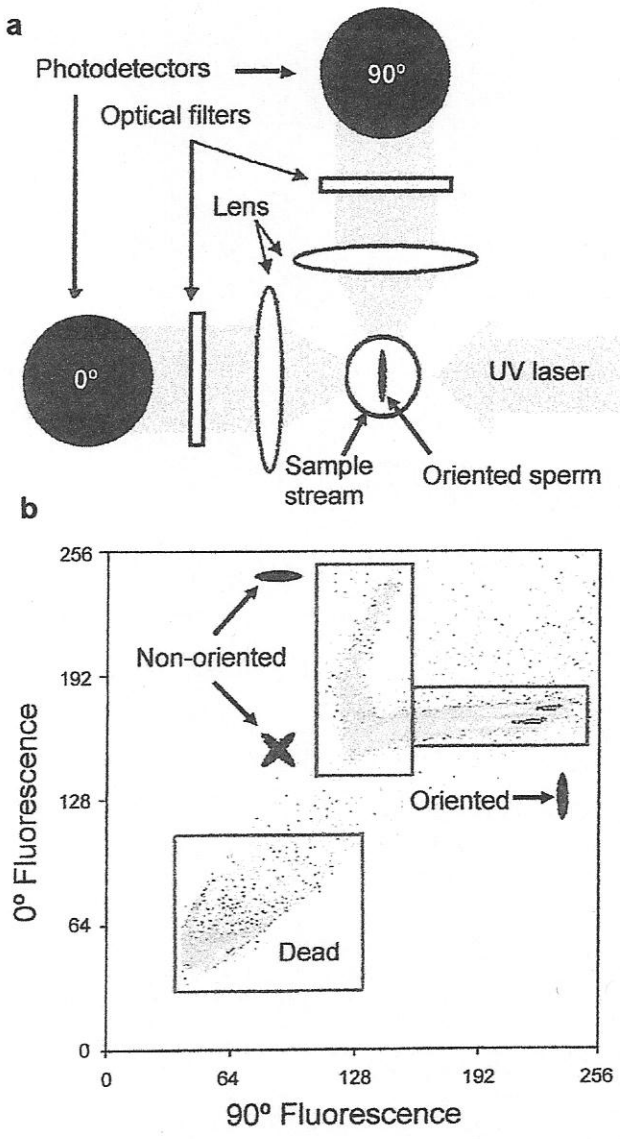

Fig. 4. Excitation and detection scheme for the flow cytometric sexing of sperm showing sperm orientation. (**a**) Configuration of laser and detectors to provide accurate measurement of DNA content of living sperm. (**b**) Bivariate histogram produced by flow cytometry showing the fluorescence measurement of live-oriented sperm with the edge toward the 90° detector (Region R1) and non-oriented sperm where the edge is toward the 0° detector or somewhere in between (R2) along with the sperm where the red food dye quenches the Hoechst 33342-stained sperm revealing the dead or damaged sperm (R3). The orientations of the sperm heads are provided adjacent to each population (below R1, for oriented sperm) and left of R2 for incorrectly oriented sperm heads Adapted from Sharpe and Evans 2009 (48).

beaker containing room temperature (~18–20°C) water prior to placing the beaker in a 5°C cold room for approximately 1.5 h. Twelve percent glycerol is added in two equal portions 15 min apart. The cooled sperm sample is then centrifuged at 5°C at $850 \times g$ for 20 min to concentrate the sperm. The supernatant is removed carefully by pouring off, leaving a 400–500 μL sperm

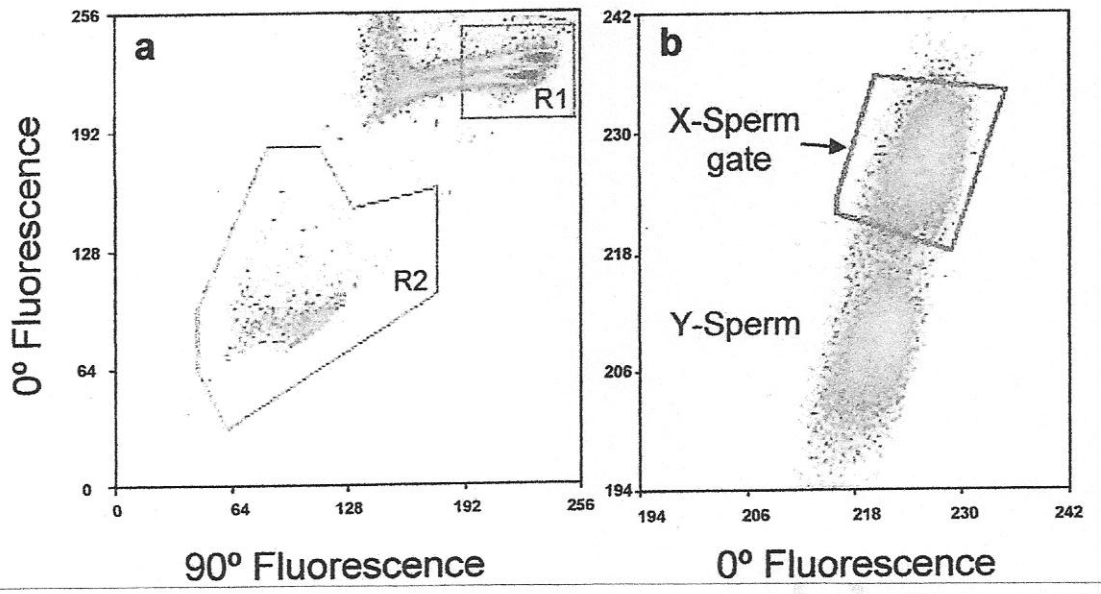

Fig. 5. An example of how the various sperm populations are gated for sorting bovine X-sperm. (**a**) Bivariate plot of 90° (*x*-axis) and 0° (*y*-axis) of the fluorescence emitted from Hoechst 3342-stained bovine sperm where properly oriented sperm are displayed in R1 and dead or damaged sperm are displayed in R2 (uptake of red food dye), (**b**) Bivariate plot of the sperm populations, X- and Y-sperm showing the gate (*box*) set for X-sperm sorting. Adapted from Sharpe and Evans 2009 (48).

pellet containing >90% of the sorted sperm. Additional Tris extender containing 20% egg yolk and 6% glycerol is added, and the sperm pellet is resuspended carefully to minimize introduction of air into the sample. This results in a final concentration of approximately 20% egg yolk and a sperm concentration of approximately 9.8×10^6/mL. The cooled sperm are examined for actual sperm concentration and for the percentage of progressively motile sperm. If the sex-sorted sperm sample exhibits a minimum of 70% progressive motility, they are then packaged at a concentration of 10×10^6 sperm/mL which results in a dose of 2.1×10^6 sperm per straw in a volume of 210 μL. The sperm are packaged into the 0.25 mL French straws and then cryopreserved.

2.8. Cryopreservation of Flow-Sorted Sperm

Cryopreservation of sexed sperm has been performed by procedures in routine use for unsexed sperm (24); however, 0.25-mL plastic straws are used instead of 0.5-mL straws, the more common packaging container for cryopreserving bovine sperm in North America. The typical sperm concentration is 10^7 sperm/mL, and the actual volume of liquid is about 0.21 mL due to the seals at each end of the straw.

The most commonly used medium for cryopreservation of sexed bovine sperm is a 20% egg yolk-Tris-based extender with 6% glycerol (v/v) as explained above and described by Schenk et al. (24). Important principles not to be violated include slow cooling over about 90 min from ambient temperature to ~5°C to prevent cold

shock, and then holding the sperm at ~5°C for 3–6 h allowing them to adjust to this low temperature before freezing; the 18 h holding at 5°C sometimes used for unsexed sperm is too long (24).

The actual freezing procedure can be done in a variety of ways as long as cooling rates are carefully controlled. The simplest and most common approach is to place straws horizontally in racks positioned a few centimeters above a pool of liquid nitrogen (which constitutes static liquid nitrogen vapor mixed with a bit of air) for 20 min or more, and then plunging the straws into liquid nitrogen (−196°C). Straws then usually are packaged into plastic goblets clipped to metal canes and stored indefinitely in liquid nitrogen tanks in the liquid or vapor phases.

Thawing of sexed sperm also is done by conventional procedures, placing the straws into a 35–37°C water bath for at least 30 s within <3 s of removing straws from the liquid nitrogen container. Straws are then ready for use for AI, IVF, or quality control purposes. The latter is essential for each batch frozen, and typically consists of evaluation of post-thaw progressive sperm motility and cell membrane integrity. Accuracy of sexing can be evaluated by resort analysis (23) continually during sorting, in a batch prior to cryopreservation, or post-thaw after processing to remove most of the egg yolk. A few percent of batches of sexed bull sperm must be discarded due to poor sperm viability post-thaw, as well as occasional batches that do not meet accuracy standards, typically at least 87% of the desired sex.

Cryopreservation procedures have been described for bovine sperm, which constitute >90% of sperm sexed commercially; species-specific variations are used as appropriate, e.g., for sheep (29) or men (30).

3. Advances in Flow Sorting of Sperm

Improvements have been made in the detector circuitry of sperm sorters, which allow the fluorescence detectors to operate at voltages lower than typically used in flow sorting. The lower detector voltage provides an improved signal to noise ratio by making the detectors nonlinear in their response, thereby creating more distance between the means of X- and Y-sperm populations (Evans et al., US Patent 7,371,517 B2). Another important advancement was development of beam shaping lenses to minimize the time that a sperm is in the laser beam path. The laser path is shaped from a round beam path to a thin elliptical, ribbon-like beam using special lenses such that the time that a sperm is irradiated at 355 nm is reduced, thus minimizing the exposure of the sperm to potential UV irradiation damage.

3.1. Damage to Sperm During the Sorting Process

Sex-sorting mammalian sperm using flow cytometry requires several steps that have the capability of damaging the gametes. Initial approaches using sperm motility evaluations to determine which steps in the process such as the mechanical aspects of sorting and centrifugation, as well as the combination of dye and laser exposure either singularly or in combination, had the potential to damage sperm were not definitive. However, flow cytometric assessment of the proportion of viable sperm at each step in the process revealed that the most damaging aspect of the process was the mechanical stress of sorting (31, 32). This problem was partially overcome by reducing the sheath pressure of the sorter from 50 to 40 psi (32).

3.2. Pulsed Laser

Significant improvements were made with the implementation of pulsed lasers compared to the original water-cooled continuous wave (CW) versions. These older CW lasers operate with a continuous beam as contrasted to the pulsed version, which pulses several hundred times as a sperm traverses the path of the beam. The Pulsed NdYag: Quazi-CW Mode Tripled laser, which emits at 355 nm, has proven to be a reliable, safe, and effective illumination system for sorting sperm. Pulsed lasers can operate for as long as 25,000 h with minimal interaction before any maintenance requirements, making the operating cost 1/10th that of the earlier water-cooled lasers. Furthermore, a pulsed laser beam can be readily split such that two or more sperm sorters can be operated simultaneously from one laser. Operation of the detector system under this pulsation not only provides lower background radiation, but also improves resolution and life span of the laser. Pulsed lasers are air cooled thereby eliminating the need for the expensive and cumbersome water cooling systems that were necessary with the CW lasers that were previously used with the sperm sorting system (33).

3.3. Digital Output

Recent research efforts have focused on innovations to increase production speeds through improved hardware, optics, and electronics. The original MoFlo® SX instrument was an analog-based system that is limited by coincidence problems such as when two sperm flow through the instrument in close proximity. This coincidence leads to discarding both sperm because the instrument cannot differentiate between the two events when two sperm are in the laser beam at the same time. A newer version of the sperm sorter, the MoFlo® SX XDP (see Fig. 3), which utilizes digital technology to overcome this coincidence problem, was recently developed through collaboration of Sexing Technologies (Navasota, TX, USA) and Beckman Coulter instruments (Beckman Coulter, Hialeah, FL and Fullerton, CA, USA). The processing electronics of the digital-based MoFlo® SX XDP can differentiate between two closely positioned sperm such that sorting yields are greatly improved. Thus, technological improvements in the current sorters

provide the ability to sort up to 10,000 X- or Y-sperm/s while achieving 90% or greater purities. The speed of sorting also is dependent on the quality of the sperm sample that is to be sorted and type of product being produced. There are now two sexed semen products available to the market 75–86% product and 87–98% product.

3.4. Profile Examples of Flow-Sorted Sperm from Different Species

Among mammalian species, both the shape of the sperm head and the X–Y sperm DNA content difference contribute to the effectiveness of utilizing DNA content to sex sort sperm (34). Although the univariate profiles of the X- and Y-sperm populations of six of the domesticated species that have been successfully sorted for predetermination of sex are similar (see Fig. 6), their DNA content differences vary somewhat; 3.8% for cattle (*Bos taurus*), 4.1% for horses (*Equus caballus*), 3.6% for swine (*Sus scrofa*), 4.2% for sheep (*Ovis aries*), 4.2% for domestic cats (*Felis catus*), and 4.4% for White-Tailed Deer (*Odocoileus virginianus*) (34–36).

3.5. Purity of Flow-Sorted Sperm Samples

A small analytical flow cytometer, the STS Purity Analyzer, was developed by Sexing Technologies to determine the purity of sorted samples, thereby eliminating the need to use the high demand sperm sorters for such quality control analyses (33, 37). This instrument assists operators in maintaining and optimizing the purity of sorted sperm by providing continual monitoring the X- or Y-sperm populations during the actual sorting process. Use of this analytical

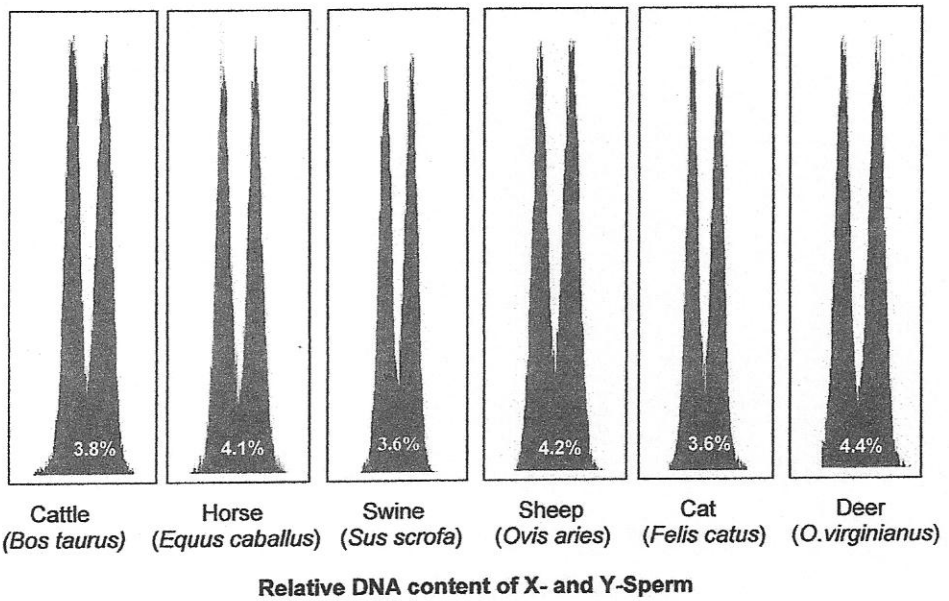

Fig. 6. Examples of univariate fluorescence profiles of Hoechst 33342-stained X- and Y-sperm populations from cattle (*Bos taurus*), horses (*Equus caballus*), swine (*Sus scrofa*), sheep (*Ovis aries*), cats (*Felis catus*), and White Tail deer (*Odocoileus virginianus*) showing the relative DNA content differences the two sperm populations for each species.

instrument as a routine monitoring effort considerably decreased production batch losses due to the failing sort purity (33, 37). Efficiency of producing sexed sperm is enhanced by setting the sort purity to approximately 90% rather than trying to achieve maximum purity of sexed sperm (see Fig. 7).

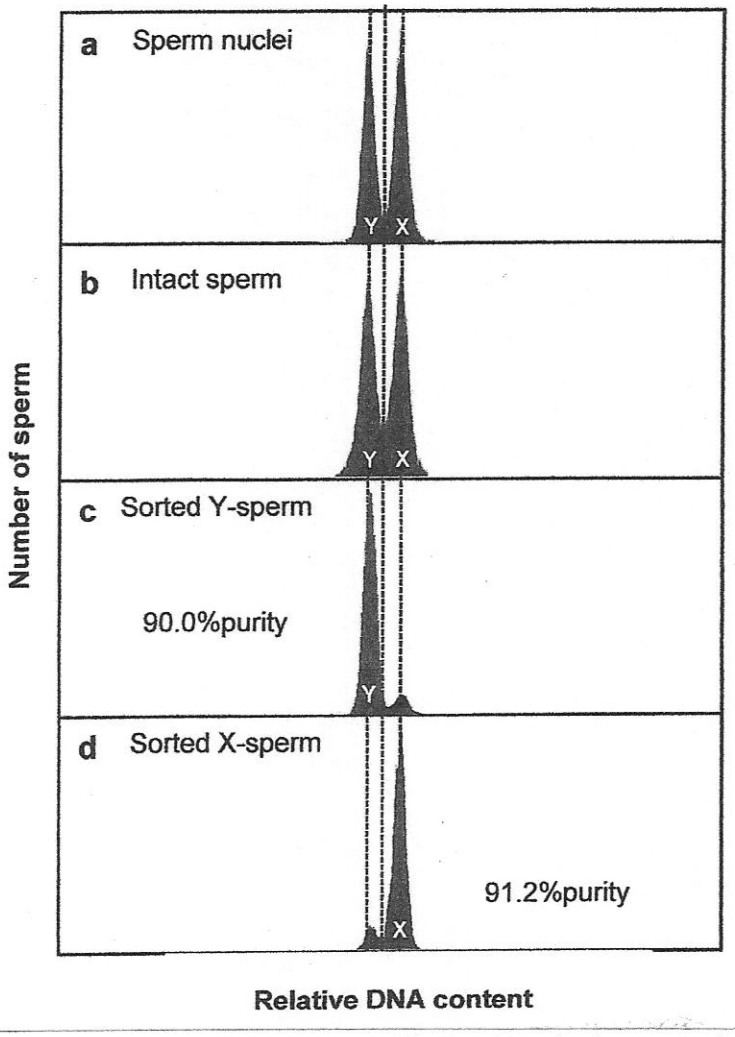

Fig. 7. Illustration of purity analyses of sex-sorted bovine sperm showing a comparison of the relative DNA content of Hoechst 33342-stained bovine sperm nuclei, living X- and Y-sperm, the Y-sorted sperm population, and sperm recovered from the X-sorted population. (a) Example illustration of the 3.8% DNA content difference between X- and Y-sperm nuclei; (b) Example of the relative DNA content difference of intact living bovine sperm stained with Hoechst 33342; (c) Representation of the frequency distribution of DNA content from reanalysis of living sex-sorted bovine Y-sperm prepared by thawing the sperm sample and removing the egg yolk-based medium by aspiration following centrifugation and restaining the sperm with Hoechst 33342; (d) Representation of the frequency distribution of DNA content from reanalysis of living sex-sorted bovine X-sperm prepared by thawing the sperm sample and removing the egg yolk-based medium by aspiration following centrifugation and restaining the sperm with Hoechst 33342. This particular example, which was sorted at relatively high sort rates, shows purities of 90.0% Y-sperm and 91.2% X-sperm for these sorted populations of living sperm.

3.6. Fertility of Sex-Sorted Mammalian Sperm

Mammalian sperm normally are available in the huge numbers required for applications such as AI. However, the process of sex-sorting sperm constitutes a bottleneck, limiting numbers of sorted sperm available for the assisted reproduction techniques required to apply sex-sorting sperm, such as IVF and AI.

The first evidence of the fertility of sex-sorted sperm was demonstrated by surgical insemination of small numbers of sperm into the reproductive tract of the rabbit, close to the normal in vivo site of fertilization (17). However, the first attempts to apply this technology were via IVF because many fewer sperm are needed for this approach than for AI (20, 21); IVF also is used routinely for human applications (30) and has been used to a limited extent with cattle. However, higher concentrations of sex-sorted sperm are needed to achieve equivalent fertilization rates in vitro than for unsorted sperm, and there are huge male-to-male differences (38, 39) with unacceptably low fertilization rates for some bulls.

Artificial insemination is a much more practical way to apply sexed semen than IVF, but requires many more sperm. Despite huge advances in flow cytometry/cell sorting, it still is impractical and uneconomical to provide $\geq 10^7$ sexed bovine sperm per insemination dose, which would be the typical dose for unsexed sperm. The compromise has been to use $\sim 2 \times 10^6$ sexed sperm per insemination dose for cattle. For horses and pigs, with typical unsexed insemination doses >100 and $1{,}000 \times 10^6$ sperm, respectively, it has thus far remained impractical to apply sexed semen commercially.

With good animal management, pregnancy rates with 2×10^6 sexed sperm per dose in cattle have usually been 70–90% of the standard $\geq 10 \times 10^6$ unsexed sperm per dose. This low dose product has been favorably received commercially, despite the lower fertility. Attempts have been made to determine if fertility is compromised mainly due to the lower sperm numbers per dose or damage to sperm during sorting. Frijters et al. (40) concluded that about one-third of the lower fertility was due to fewer sperm. However, with rare exceptions (41), it has not been possible to compensate for the reduced fertility with sexed sperm by increasing the sperm dose (42–46). Research is continuing to address this problem.

3.7. Commercial Development

The flow cytometric sperm sexing technology described above has been utilized commercially in cattle (*Bos taurus and Bos indicus*) for nearly 10 years resulting in more than 10 million live births of calves of a predetermined sex. Sperm from a variety of other mammalian species including horses (*Equus caballus*), boars (*Sus scrofa*), rams (*Ovis aries*), dogs (*Canis lupis familiaris*), cats (*Felis catus*), elk (*Cervus canadiensis*), water buffalo (*Bubalus bubalis*), and White-Tail deer (*Odocoileus virginianus*) have been successfully sex sorted and inseminated resulting in offspring of a predetermined sex (33–36, 45, 47). The application of this sex-sorting technology to humans by Genetics and IVF Institute, Fairfax, VA has resulted in more than 1,000 babies (30).

Acknowledgments

The authors are grateful to Rick Lenz for his suggestions and to Justine O'Brien and David Del Olmo Llanos for providing fluorescence profile data.

References

1. Moruzzi JF (1979) Selecting a mammalian species for the separation of X- and Y-chromosome-bearing spermatozoa. J Reprod Fertil 57:319–323
2. Sumner AT, Robinson JA (1976) A difference in dry mass between the heads of X- and Y-bearing human spermatozoa. J Reprod Fertil 48:9–15
3. Gledhill BL et al (1982) Identifying X- and Y-chromosome-bearing sperm by DNA content: retrospective perspective and prospective opinions. In: Prospects for sexing mammalian sperm. Colorado Associated University Press, Boulder, CO, pp 177–191
4. Van Dilla MA et al (1977) Measurement of mammalian sperm deoxyribonucleic acid by flow cytometry. Problems and approaches. J Histochem Cytochem 25:763–773
5. Meistrich ML et al (1979) Resolution of x and y spermatids by pulse cytophotometry. Nature 274:821–823
6. Otto FJ et al (1979) Flow cytometry of human spermatozoa. Histochemistry 61:249–254
7. Gohde W (1973) Automation of cytofluorometry by use of the impulse microphotometer. In: Thaer AA, Sernetz M (eds) Fluorescence techniques in cell biology. Springer, Berlin, pp 79–88
8. Fulwyler MJ (1977) Hydrodynamic orientation of cells. J Histochem Cytochem 25:781–783
9. Pinkel D et al (1982) High resolution DNA content measurements of mammalian sperm. Cytometry 3:1–9
10. Cattanach BM (1961) A chemically-induced variegated-type position effect in the mouse. Z Vererbungsl 92:165–182
11. Garner DL et al (1983) Quantification of the X- and Y-chromosome-bearing sperm of domestic animals by flow cytometry. Biol Reprod 28:312–321
12. Kamentsky LA et al (1963) Ultraviolet absorption in epidermoid cancer cells. Science 142:1580–1583
13. Kamentsky LA et al (1965) Spectrophotometer: new instrument for ultrarapid cell analysis. Science 150:630–631
14. Herzenberg LA et al (1976) Fluorescence-activated cell sorting. Sci Am 234:108–117
15. Pinkel D et al (1982) Sex preselection in mammals? Separation of sperm bearing Y and "O" chromosomes in the vole *Microtus oregoni*. Science 218:904–906
16. Johnson LA, Pinkel D (1986) Modification of a laser-based flow cytometer for high-resolution DNA analysis of mammalian spermatozoa. Cytometry 7:268–273
17. Johnson LA et al (1989) Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod 41:199–203
18. Johnson LA et al (1987) Flow sorting of X and Y chromosome-bearing spermatozoa into two populations. Gamete Res 16:1–9
19. Johnson LA (1991) Sex preselection in swine: altered sex ratios in offspring following surgical insemination of flow sorted X- and Y-bearing sperm. Reprod Dom Anim 309–314
20. Cran DG et al (1993) Production of bovine calves following separation of X- and Y-chromosome bearing sperm and in vitro fertilisation. Vet Rec 132:40–41
21. Cran DG et al (1995) Sex preselection in cattle: a field trial. Vet Rec 136:495–496
22. Johnson LA et al (1994) Improved flow sorting resolution of X- & Y-chromosome bearing viable sperm separation using dual staining and dead cell sorting. Cytometry Suppl 7: Abstr 476D
23. Johnson LA, Welch GR (1999) Sex preselection: high-speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenology 52:1323–1341
24. Schenk JL et al (1999) Cryopreservation of flow-sorted bovine spermatozoa. Theriogenology 52:1375–1391
25. Peters D et al (1985) The LLNL high-speed sorter: design features, operational characteristics, and biological utility. Cytometry 6:290–301
26. van den Engh G, Stokdijk W (1989) Parallel processing data acquisition system for multilaser flow cytometry and cell sorting. Cytometry 10:282–293

27. Rens W et al (1998) A novel nozzle for more efficient sperm orientation to improve sorting efficiency of X and Y chromosome-bearing sperm. Cytometry 33:476–481
28. Rens W et al (1999) Improved flow cytometric sorting of X- and Y-chromosome bearing sperm: substantial increase in yield of sexed semen. Mol Reprod Dev 50–56
29. de Graaf SP et al (2007) Birth of offspring of pre-determined sex after artificial insemination of frozen-thawed, sex-sorted and re-frozen-thawed ram spermatozoa. Theriogenology 67:391–398
30. Karabinus DS (2009) Flow cytometric sorting of human sperm: MicroSort clinical trial update. Theriogenology 71:74–79
31. Garner DL, Suh TK (2002) Effect of Hoechst 33342 staining and laser illumination of viability of sex-sorted bovine sperm. Theriogenology 746 (Abstr)
32. Suh TK et al (2005) High pressure flow cytometric sorting damages sperm. Theriogenology 64:1035–1048
33. Evans KM(2010) Interpretation of sex-sorting process and new developments. In: Proceedings of the 23rd technical conference on artificial insemination & reproduction, National Association of Animal Breeders, pp 93–98
34. Garner DL (2006) Flow cytometric sexing of mammalian sperm. Theriogenology 65: 943–957
35. Garner DL, Seidel GE Jr (2008) History of commercializing sexed semen for cattle. Theriogenology 69:886–895
36. O'Brien JK et al (2009) Application of sperm sorting and associated reproductive technology for wildlife management and conservation. Theriogenology 71:98–107
37. Kjelland ME et al (in press) Sex selection technology and sperm DNA fragmentation. In: Ridha-Albarzanchi M, Dukelow RW, Alanssari SAM (eds) Primate reproduction, IVF and assisted reproductive technology
38. Xu J et al (2009) Optimizing IVF with sexed sperm in cattle. Theriogenology 71:39–47
39. Barcelo-Fimbres M et al (2011) In vitro fertilization using non-sexed and sexed bovine sperm: sperm concentration, sorter pressure, and bull effects. Reprod Domest Anim 46: 495–502
40. Frijters AC et al (2009) What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? Theriogenology 71:64–67
41. Schenk JL et al (2009) Pregnancy rates in heifers and cows with cryopreserved sexed sperm: effects of sperm numbers per inseminate, sorting pressure and sperm storage before sorting. Theriogenology 71:717–728
42. Seidel GE Jr, Schenk JL (2008) Pregnancy rates in cattle with cryopreserved sexed sperm: effects of sperm numbers per inseminate and site of sperm deposition. Anim Reprod Sci 105:129–138
43. DeJarnette JM et al (2008) Effect of sex-sorted sperm dosage on conception rates in Holstein heifers and lactating cows. J Dairy Sci 91:1778–1785
44. DeJarnette JM et al (2010) Effects of 2.1 and 3.5 × 10(6) sex-sorted sperm dosages on conception rates of Holstein cows and heifers. J Dairy Sci 93:4079–4085
45. Dejarnette JM et al (2011) Effects of sex-sorting and sperm dosage on conception rates of Holstein heifers: is comparable fertility of sex-sorted and conventional semen plausible? J Dairy Sci 94:3477–3483
46. Garner DL (2001) Sex-Sorting mammalian sperm: concept to application in animals. J Androl 22:519–526
47. DeYoung RW et al (2004) Do *Odocoileus virginianus* males produce Y-chromosome-based ejaculates? Implications for adaptive sex ration theories. J Mammal 85:768–773
48. Sharpe JC, Evans KM (2009) Advances in flow cytometry for sperm sexing. Theriogenology 71:4–10