



Sex - Sorted semen Technology, Application and Future Perspectives in Livestock

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Abstract

Sex-sorted semen technology is an important advancement in reproductive biotechnology that enables the production of offspring of a predetermined sex in livestock. In dairy cattle and buffalo production, this technology has particular significance because it can increase the availability of genetically superior replacement females, accelerate herd improvement, and reduce the production of unwanted male calves in dairy-oriented systems. The biological basis of sperm sexing is the difference in DNA content between X- and Y-chromosome-bearing spermatozoa, with X-bearing sperm containing approximately 3.8–4.2% more DNA than Y-bearing sperm in ruminants. Fluorescence-activated cell sorting (FACS), using DNA-binding fluorochromes such as Hoechst 33342, remains the principal commercially validated method for producing sex-sorted semen with high sex-purity. Although conventional physical separation approaches based on density, motility, sedimentation, or surface characteristics have been investigated, their reliability for commercial applications remains limited. Recent developments in flow-cytometric sorting have focused on improving sperm recovery, reducing cellular stress, increasing sorting efficiency, and enhancing post-thaw fertility. At the same time, emerging technologies, including microfluidic and acoustofluidic separation, immunological targeting, TLR7/8-mediated sperm selection, and nanoparticle-assisted approaches, are being explored as potential label-free or minimally invasive alternatives. In cattle and buffalo production, sex-sorted semen can contribute to genetic improvement, reproductive efficiency, herd replacement planning, and improved economic sustainability when appropriately integrated with artificial insemination, genomic selection, fixed-time AI, and embryo production programmes. However, fertility, conception rate, sperm dose, animal factors, semen-handling procedures, and production costs remain important considerations. Continued refinement of sorting technologies and integration with precision breeding and reproductive management could further expand the application of sex-preselection technology in livestock production.

Keywords: Sex-sorted semen; Sperm sexing; Sex preselection; X-bearing sperm; Y-bearing sperm; Flow cytometry; FACS; Hoechst 33342; Artificial insemination; Dairy cattle; Buffalo; Reproductive biotechnology; Microfluidics; Acoustofluidics; TLR7/8; Genetic improvement.

Introduction

The ability to predetermine the sex of offspring prior to conception represents one of the most transformative advances in modern animal biotechnology and reproductive physiology. In dairy and beef cattle production systems, sex preselection directly addresses key economic and operational inefficiencies. Historically, unselected breeding results in a 1:1 male-to-female sex ratio, producing an excess of male calves that often possess limited zoo-technical or market value in dairy operations. The strategic application of sex-sorted semen—containing predetermined proportions of X- or Y-chromosome-bearing spermatozoa—enables producers to bias offspring ratios up to 90% toward the desired gender, dramatically increasing the availability of high-value replacement heifers or specialized beef progeny.

At the physiological level, mammalian sex sorting relies on fundamental differences between X- and Y-bearing spermatozoa. Because the X-chromosome is significantly larger than the Y-chromosome, X-sperm contain approximately 3.8% to 4.2% more total genomic DNA (depending on the species). The primary gold standard for commercial sperm sexing is Fluorescence-Activated Cell Sorting (FACS) combined with flow cytometry. Spermatozoa are stained with a DNA-binding fluorophore (such as Hoechst 33342), exposed to laser excitation, and separated based on differential fluorescence intensity output. The identified cells are subsequently given electrostatic charges and deflected into discrete collection channels.

Biological Basis of Sperm Sexing

The biological foundation of mammalian sperm sexing relies primarily on the physical and genomic disparities between X- and Y-chromosome-bearing spermatozoa (He et al., 2022). Following meiotic division during spermatogenesis, mature spermatozoa package either an X or a Y sex chromosome. Because the mammalian X chromosome is substantially larger and contains more nucleotide base pairs than the Y chromosome, X-bearing spermatozoa possess a higher total

genomic DNA content. In ruminant livestock, X-sperm contain approximately 3.8% to 4.2% more DNA than Y-sperm—such as ~3.8% in bulls and up to ~4.2% in rams and bucks (Ngcobo et al., 2024; Rahman & Pang, 2020). This measurable difference in nucleic acid mass serves as the primary biophysical parameter for high-resolution flow cytometric sorting, where fluorophore uptake (e.g., Hoechst 33342) is directly proportional to DNA mass (Morrell et al., 1988).

Technologies and method of semen sexing

Over the past few decades, numerous physical, biological, and technological strategies have been developed to separate X- and Y-chromosome-bearing spermatozoa. While several novel methodologies are currently under investigation to reduce cellular damage and processing costs, flow cytometric sorting remains the only commercially validated technology that achieves high-purity (>90%) sex-preselection in livestock.

1. High-Throughput Flow Cytometry (FACS)

Flow-cytometric separation—originally based on the Beltsville Sperm Sexing Technology—relies on quantifying nuclear DNA mass differences using a fluorophore. Spermatozoa are incubated with Hoechst 33342, a membrane-permeable bis-benzimidazole dye that binds stoichiometrically to the minor groove of double-stranded DNA. As individual cells pass in single file through an ultraviolet (UV) or violet semiconductor laser, the higher DNA content of X-bearing sperm yields a proportionally brighter fluorescent emission.

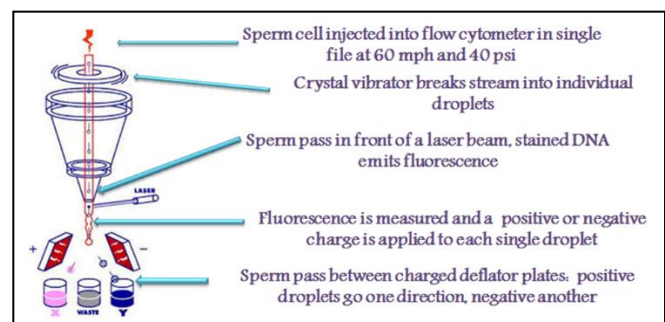


Diagram of high-Throughput Flow Cytometry (FACS)

Modern Refinements (e.g., SexedULTRA™): Recent developments have addressed historic drawbacks by replacing harsh UV lasers with gentler pulsed lasers, optimizing sheath fluid osmolarity, reducing hydro-dynamic pressure, and increasing sorting speeds (up to 40,000 events/second). These modifications significantly diminish cellular stress and boost straw dosage thresholds.

2. Physical & Density Gradient Methods

Earlier non-cytometric methods sought to exploit hypothesized physical differences between X and Y sperm, including sedimentation rate, volumetric size, surface charge, and swimming velocity.

Percoll & Bovine Serum Albumin (BSA) Gradients: Density gradient centrifugation relies on the premise that heavier X-sperm sediment faster through discontinuous gradients.

Swim-Up Techniques: Modified swim-up assays operate on the assumption that lighter Y-sperm display faster forward progressive motility.

While these methods are simple, low-cost, and non-damaging to sperm membranes, recent proteomic and morphological validation studies demonstrate that they yield inconsistent sex-purity ratios (often failing to exceed 60–65%). Consequently, physical separation methods are generally deemed unsuitable for commercial livestock breeding applications.

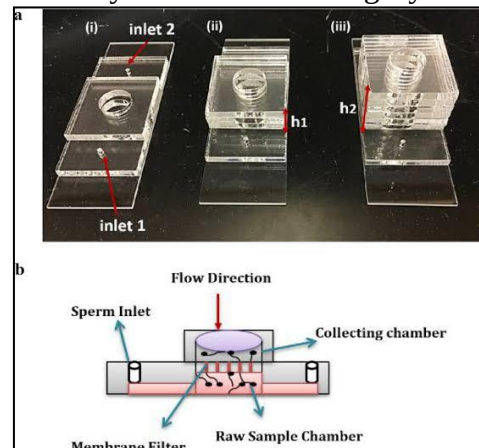
3. Emerging Microfluidic and Acoustofluidic Sorting

To overcome the physical stressors associated with conventional flow cytometry (high pressure, laser damage, and electrostatic shock), microfluidic technology has emerged as a promising alternative. Microfluidic platforms utilize microscale channel architectures to manipulate cells via passive hydrodynamic forces or active acoustic fields (acoustofluidics).

Motility and Hydrodynamic Selection: Microfluidic chips can sort sperm based on rheotaxis (movement against fluid flow) or

differential migration in viscous media without subjecting cells to shear stress.

Acoustic Wave Separation: Surface acoustic waves (SAW) applied across microchannels exert differential acoustic radiation forces on sperm based on subtle variations in cell volume and density. Microfluidic sorting operates under low pressure, eliminates the need for fluorescent dyes, and maintains higher post-sort motility and structural integrity.



Microfluidic device for sorting functional sperm

4. Immunological and Nanoparticle-Based Sexing

Immunological targeting relies on identifying sex-chromosome-specific surface markers or receptors uniquely expressed on either X- or Y-bearing spermatozoa.

Targeting Toll-Like Receptors (TLR7/8): Recent functional studies identified that genes encoding endosomal receptors TLR7 and TLR8 are expressed exclusively in X-bearing sperm. Ligand-binding activation of TLR7/8 selectively suppresses mitochondrial ATP production and swimming velocity in X-sperm without impairing Y-sperm, enabling motility-based separation.

Nanoparticle-Mediated Immunomagnetic Sorting: Functionalized magnetic nanoparticles coated with X- or Y-specific antibodies (or lectins) selectively bind to target sperm populations. The sample is passed through a magnetic field, retaining the bound population while the unbound fraction flows through uninhibited.

Although immunological and microfluidic technologies are still undergoing field-validation, they represent the next generation of semen sexing—promising high-throughput, dye-free, and cost-effective alternatives to flow cytometry.

Applications of Sex-Sorted Semen in Cattle and Buffalo Production

1. Acceleration of Genetic Progress and Herd Expansion

Historically, genetic progress in dairy herds was heavily constrained by slow dam-pathway selection, as nearly 90% of genetic progress relied on sire selection. Incorporating sex-sorted semen allows producers to selectively breed only top-tier genetic females to produce replacement heifers.

2. Animal Health, Welfare, and Environmental Impacts

The targeted utilization of X-chromosome-bearing semen delivers clear health, welfare, and environmental benefits:

Reduction of Dystocia

Mitigation of Male Calf Welfare Issues

Mitigating Environmental Footprints

3. Specific Applications and Nuances in Buffalo (*Bubalus bubalis*) Production

Water buffaloes serve as vital dairy animals in tropical and subtropical regions, particularly across South Asia and the Mediterranean. However, sex-sorted semen applications in buffalo farming encounter unique biological and logistical challenges:

Addressing Male Calf Mortality

Genetic Upgrading of Low-Yielding Herds

Reproductive Physiology & Timed AI (TAI)

Recent advances and future perspective

Recent technological advancements in sperm sexing have concentrated primarily on narrowing the historical fertility gap between sex-sorted and conventional non-sorted semen. Innovations such as high-concentration sorting platforms (e.g., SexedULTRA 4M™), dye formulation optimizations, low-pressure fluidics, and gentle pulsed laser illumination have significantly reduced sorting-induced oxidative stress, physical shearing, and premature capacitation. Consequently, field conception rates using high-dose sexed semen

are approaching parity with conventional insemination protocols, particularly when integrated into fixed-time artificial insemination (FTAI) and *in vitro* embryo production (IVP) pipelines.

Looking toward future prospects, the field is shifting away from traditional flow cytometry toward label-free, dye-free, and high-throughput technologies. Emerging platforms leverage acoustofluidic microfluidics to sort cells based on subtle volumetric dynamics without cellular injury, microRNA and proteomic biomarker profiling to selectively label sex-specific membrane receptors (e.g., TLR7/8 targeting), and magnetic nanoparticle immunoseparation. Concurrently, combining non-invasive sexed semen methods with genomic selection and machine-learning-assisted sperm diagnostics promises to further accelerate genetic gain, eliminate male calf surplus welfare issues, and expand the scalable adoption of sex-preselection technology across a broader spectrum of global livestock species.