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Advances in flow cytometry for sperm sexing

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Abstract

This review presents the key technological developments that have been implemented in the 20 years since the first reports of successful measurement, sorting, insemination and live births using flow cytometry as a proven physical sperm separation technique. Since the first reports of sexed sperm, flow technology efforts have been largely focused on improving sample throughput by increasing the rate at which sperm are introduced to the sorter, and on improving measurement resolution, which has increased the proportion of cells that can be reliably measured and sorted. Today, routine high-purity sorting of X- or Y-chromosome-bearing sperm can be achieved at rates up to 8000 s^{-1} for an input rate of 40,000 X- and Y- sperm s^{-1}. With current protocols, straws of sex-sorted sperm intended for use in artificial insemination contain approximately 2×10^6 sperm. The sort rate of 8000 sperm s^{-1} mentioned above corresponds to a production capacity of approximately 14 straws of each sex per hour per instrument.

Keywords: Gender preselection; Sperm sexing; Sperm sorting; Flow cytometry; Sort rate; Sort efficiency

1. Introduction

Since the first reported analysis of sperm on a flow cytometer [1,2] there has been significant interest in developing a robust technique to sort based on DNA content. The first sorting of X- and Y-chromosome-bearing sperm [3–5], and subsequent proven statistical alteration of the sex of the offspring by inseminating those sperm [6] was carried out using a modified jet-in-air flow cytometer, an instrument used in many scientific research applications to optically analyze and subsequently sort cells as they pass through an illumination source [7].

Sustained interest in commercializing the flow-based sperm sorting technique has ensued, as has the search for alternative approaches to sperm sexing and sex pre-selection [8]. However despite these efforts no other technique has proven to be as effective to date as flow sorting [9]. As such, the flow sorting technique is now routinely used in a number of commercial sperm sexing facilities for various breeding purposes, particularly in bovine applications [10]. It is expected that four million straws of sexed bovine sperm will be produced in the 2008 calendar year, up from a total of two million straws in 2007. There also have been a number of reports on the use of the technique in other species [11–17].

Flow cytometry-based sperm sexing has undergone a number of improvements in throughput and sort efficiency (with a goal toward commercial viability of the technique) since first being implemented 20 years ago. In the current approach, known as the Beltsville Sperm Sexing Technology [18], sperm are prepared with a DNA-specific stain (Hoechst 33342) and excited by a UV laser for DNA content measurement. A quencher dye (red food coloring) is also added during sample preparation to identify dead (membrane-damaged) cells

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so that only live sperm are sorted [19]. Current state-of-the-art instruments at Sexing Technologies facilities use a solid-state laser for UV excitation, dual orthogonal detectors (at 0° and 90° to the laser), an orienting nozzle, and digital electronics to provide sorted subpopulations of X- or Y- bearing sperm at rates of approximately 8000 cells s^{-1} (greater than 90% pure) when operating at an input event rate of 40,000 events s^{-1}. This represents a sort efficiency of approximately 42% of available X- and Y-bearing (live and dead) sperm from an original sample with an assumed 1:1 X:Y ratio.

Several excellent articles have reviewed the reproductive aspects of the sperm sorting process [10,20] and the economic ramifications of using the technique in herd management [21,22], so these are not considered here. Alongside the technical improvements in flow cytometry, there have been significant and parallel developments in sperm physiology allowing sorted sperm to be cryopreserved and utilized for in vitro embryo production and low dose artificial insemination (with satisfactory fertility) in cattle, sheep and pigs. Procedures also have been developed to allow the sex sorting of previously frozen semen in sheep and cattle.

This report reviews the key advances in flow sorting technique that have enabled current throughput and sort efficiency levels to be achieved from only a few tens of sperm s^{-1} in early investigations. For the sperm sorting process, throughput and efficiency are governed by three key factors; measurement resolution, cell orientation, and statistical and timing aspects. These aspects and their historical developments are reviewed with potential areas for improvement in mind.

2. Flow cytometry and sperm sorting

For the purposes of this review, throughput is defined as the sort rate or number of desired sperm sorted s^{-1}, and efficiency as the proportion of cells that can be measured and physically sorted relative to the total sperm population present in a sample. The yield represents the total number of sperm sorted over a given time period, and sort recovery is the number of sperm actually collected vs. the number in droplets purportedly sorted from the main stream.

2.1. Measurement resolution

Measurement resolution, or more specifically the ability to resolve fluorescently tagged X- and Y- sperm populations that have a small total difference in DNA content, arguably influences throughput, efficiency, and sort purity to the largest extent. In the case of bovine sperm, the X–Y difference has been measured to cover the range 3.7–4.22% depending on specific breed, and from 2.3% to 7.5% for a number of other mammalian species [20]. Together with biological variation and staining quality within a sample, instrument factors can greatly impact resolution of measurement and therefore discrimination of each sperm population. These factors include fluidic instability, laser noise, electronic and photodetector noise, light collection efficiency, acoustic vibration, and even cleanliness of the fluidic lines and sheath fluids. When sperm fluorescence is measured using a flow cytometer, the differences between X- and Y-chromosome-bearing sperm are observed on a histogram as a double Gaussian distribution. An increase in coefficient of variation of the order of less than a few tenths of a percentage can have a significant effect on confidently identifying, classifying, and sorting X- and Y-sperm populations as shown in Fig. 1.

Several aspects of the flow cytometer have been improved to reduce noise and therefore increase the measurement resolution of X- and Y-sperm. Gas-based argon ion lasers have been replaced with low-noise units such as the Vanguard™ diode-pumped solid state system (Newport Corporation, Irvine, CA, USA). Alternative measurement and gating schemes that combine photodetector pulse height and area have been implemented to more clearly distinguish X- and Y-bearing sperm [23]. Software and visualization aspects

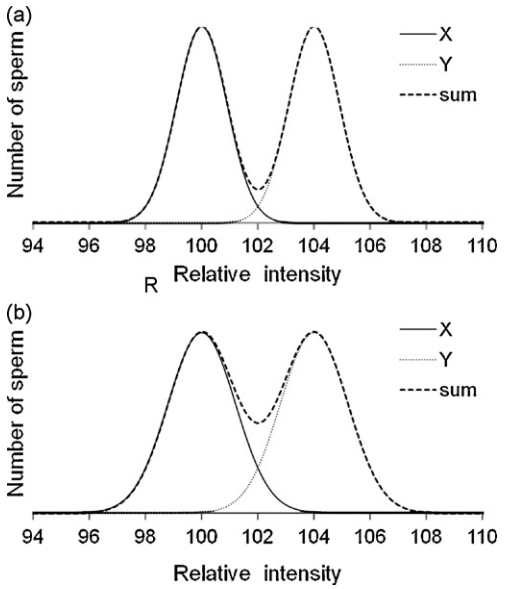

Fig. 1. Sort rates and purity can be compromised through biological and instrument factors. Simulated fluorescence data for X- and Y-sperm populations separated by 4% show an observable difference in X–Y overlap for coefficient of variation values (a) 0.9%, and (b) 1.2%.

such as data zoom, tracking (to overcome drift), and rotation functions have also been used (in a manner analogous to fluorescence compensation in multi-fluorophore immunology applications) to overcome mismatches in illumination and detection for suboptimal cell orientations. By rotating the entire bivariate, X–Y resolution is enhanced, and sort regions can be set to yield optimal sort purity. Modifications have been made to maximize fluorescence collection using high numerical aperture optical components and high transmission optical filters. Despite not being recommended by the manufacturer, resolution improvements also have been made by operating photomultiplier detection devices in a low (i.e. less than 300 V) voltage range [23].

2.2. Cell orientation

Orientation of sperm has a considerable effect on yield and throughput in flow sorting due to optical measurement artefacts arising from the aspherical shape of the sperm head in most mammalian species [1,24]. In the case of bovine sperm, these artefacts are a direct result of non-uniformities in both illumination and fluorescence for cells that flow through the excitation/detection region at arbitrary orientations around the flow axis (Fig. 2). This can result in an edge:flat face fluorescence measurement ratio of 2:1 that can be attributed to refractive index effects such as total internal reflection and light piping [1]. We have found that those sperm that are presented largely edge toward (i.e. at angles up to 45°) the excitation source are not illuminated adequately to provide clear X–Y resolution [25]. Thus, attempting to reliably measure a 4% difference in DNA content with a jet-in-air sorter is highly problematic unless cell orientation can be controlled or accounted for by some means.

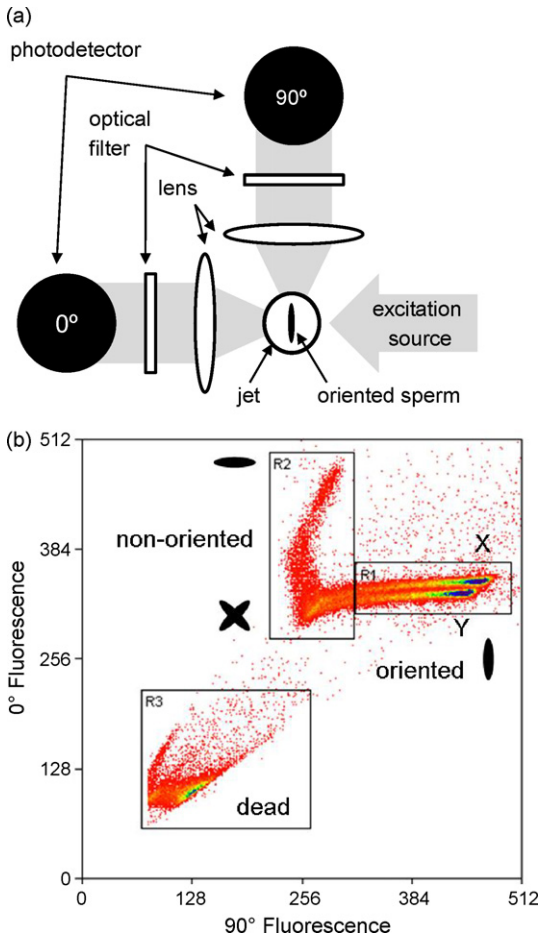

Fig. 2. (a) Excitation and detection scheme used for flow cytometric sorting of sperm showing preferred sperm orientation (flow axis into page), and (b) 0° vs. 90° fluorescence bivariate histogram produced by a flow cytometer showing the effect of approximate 2:1 fluorescence measurement for live oriented sperm that are edge toward the 90° detector (region R1), and non-oriented sperm that are edge toward the 0° detector or somewhere between (R2). X- and Y-sperm populations can be resolved within R1. Dead (red food dye quenched) sperm are present in R3.

2.2.1. Fluidic orientation techniques

Early flow cytometric sperm analysis was carried out without any type of planar alignment device, resulting in approximately 20–30% of intact and tail-less nuclei being positioned at an appropriate angle to distinguish between fluorescently tagged X- and Y-sperm populations [26]. Extending the work of others [24,27], a bevelled injection needle was introduced and shown to hydrodynamically orient approximately 60% of nuclei at low flow rates leading to sort rates of 50–150 sperm s^{-1} [28]. A desire to sort intact cells led to further improvements to the nozzle design to increase sort efficiency. Subsequently, the development of an orient-tip nozzle device doubled the proportion of live intact cells sufficiently well oriented for high resolution measurement to 60% [29,30]. Further refinements have built on this work by optimizing nozzle manufacture to a point where orientation efficiencies of 60–80% can consistently be achieved in our production facilities depending on bull and sample quality.

2.2.2. Optical techniques

Even with the aforementioned fluidic developments for sperm sorting, a remaining 20–40% of live sperm are not measurable, and pass through the flow cytometer directly to waste. A number of investigations have explored alternative optical illumination and detection

solutions to the cell orientation problem [31–33], but none of these has been implemented in routine operation to date. Recent investigations suggest that orientation efficiency can be increased beyond current levels by a further 5–15% (400–1200 sperm s^{-1}) by employing two additional photodetectors positioned at 45° and 135° relative to the 0° detector [25]. This semi-octagonal arrangement enables diagonally oriented sperm (relative to the 0° and 90° collection optics) to be measured accurately since a sperm that is oriented edge toward one detector is optimally oriented for fluorescence collection from the other (i.e. out of the flat face). We are also exploring the use of an additional laser/detector pair (oriented at 90° to the first laser axis) to capture those remaining sperm that, due to their orientation, are not sorted in any current system [25]. The successful implementation of such a method would ensure that no sperm is sent to waste on the basis of its orientation.

2.3. Statistics and timing aspects

Statistical and timing aspects also set fundamental limits to the potential rate of sperm sorting. Suitably concentrated sperm must be introduced to the sheath fluid and excitation source in single file to avoid coincidence and hard abort (or analysis coincidence) events, which restrict analysis rates. However, even if all sperm are sufficiently well spaced from one another to be resolved and measured, their asynchronous timing further downstream with respect to other cells and individual droplet formation cycles can also force soft-abort sort events, and severely limit throughput rates. In commercial settings, sperm sorters routinely operate at an operating pressure of 276 kPa (40 psi) and droplet formation frequencies of 60–70 kHz (corresponding droplet formation period of 16–14 μs respectively). A sperm traverses the excitation beam (a 20 μm tall × 160 μm wide spot) vertically at approximately 18 m s^{-1} to produce a 1.4 μs pulse. Therefore, depending on the rate of sample introduction (event rate) to the outer-flowing sheath fluid, it is possible that multiple sperm will ultimately be contained within a single droplet. If those sperm differ, by sex for example, (i.e. where a desired and an unwanted cell is within a single drop) then a soft-abort (or sort coincidence) condition exists. In general, the processing capacity of electronic acquisition and processing systems has a significant bearing on sort speeds as does the velocity and size of sperm, spot size of the excitation source, and the field of view of the photodetector system.

Early sperm sexing systems such as the FACStar (BD Biosciences, San Jose, CA, USA) and EPICS V (Beckman Coulter, Inc., Fullerton, CA, USA) series instruments, when modified slightly, performed successful sorts by analysing and sorting cells at rates of up to 100 and 150 cells s^{-1} respectively [28,34]. In the late 1990 s the introduction of the MoFlo high speed sorter (originally developed at the Lawrence Livermore National Laboratory, Livermore, CA, USA) [35] to sperm sexing provided the necessary fluidic handling and electronic processing speeds to provide increased input and sort rates approaching 1700 s^{-1} [26]. The introduction of this system also provided increased efficiency through refined droplet sorting modes that enabled similarly-classified particles (e.g. multiple X or multiple Y sperm) to be sorted when they would otherwise produce soft coincidence abort events. A purpose-built version of the LLNL flow cytometer called the MoFlo SX (Cytomation, Inc., Ft Collins, CO, USA, recently acquired by Beckman Coulter, Inc., Fullerton, CA, USA) was developed and supplied to XY, Inc. and its licensees, and was reported to be able to sort sperm at a rate of 5500 s^{-1} [20].

An important recent development in flow cytometer technology that directly affects sperm sexing throughput and efficiency is introduction use of digital pulse processing circuitry in place of time-gated analogue systems. The major advantage of digital electronics for sperm sexing, as shown in early validation studies with a MoFlo XDP (Beckman Coulter, Inc., Fullerton, CA, USA), is that measurement dead time (a temporary inability to process and resolve photodetector pulses due to closely spaced sperm), and therefore hard coincidence events, are almost completely eliminated. This is because digital systems are capable of continuous, near real-time sampling which in turn enables multiple photodetector pulses to be reliably discriminated and measured. As an example of this effect, if a given sperm sample (concentration 80×10^6 sperm ml^{-1}) is analysed at an input detection rate of 35,000 cells s^{-1} by an analogue MoFlo SX system, typical sort rates of 6000 cells s^{-1} (X and/or Y) will be observed with a hard abort rate, due to coincidence events, of approximately 4500–5000 droplets s^{-1}. When the same sample is analyzed digitally at a similar flow rate one observes an increase in the event rate to 40,000 s^{-1} (since these additional events can now be resolved and measured) and sort rate to approximately 8000 cells s^{-1}. This represents a 33% increase in throughput and yield, and an increase in sort efficiency from 30% for the analogue system (when hard coincidences are included) to over 40% of

available X- and Y-sperm. Soft abort rates are not altered with the digital system, reducing potential sort events by 1800-2000 droplets s^{-1} under normal operating conditions (i.e. sample concentration, injection rate, and droplet generation frequency).

3. Commercial drivers and sorter parallelism

Despite the developments discussed above, relatively little effort has been applied over the last decade to increase throughput (sort rates) per instrument sort channel. Instead, attention has been paid to meeting production demands through multi-instrument installations and 24 h operations, thereby increasing the sorter to sample ratio. Operational efficiency gains have also been made by reducing instrument footprint and by sharing lasers, pumps, and even labor across multiple instruments [10]. Continuous operation of sorters has led to a reduction in down time associated with instrument start-up sequences such as fluid blockages and setting sort parameters, and equilibration requirements such as laser warm-up and jet stability. The introduction of sterile pre-packaged sheath media and the removal of in-line filters have also shortened cleaning cycles at change-over (from approximately 40 to 10 min) thus increasing instrument availability for sorting.

One case in which a large amount of effort was applied was in the exploration of sort channel parallelism where four sort heads were combined in a single instrument (Monsanto Company, St. Louis, MO, USA). This system was abandoned in 2007 due to problems with field application [10]. The underlying flow cytometry technologies associated with this work were acquired recently by Sexing Technologies, Inc. (Moreno, personal communication), and several aspects of the Monsanto approach are included in the Reflection™ parallel sorting flow cytometer produced by iCyt Mission Technology, Inc. (Champaign, IL, USA). Because single channel sort efficiencies are approaching fundamental limits, the trend towards sort channel parallelism will likely continue, particularly if multiple channel systems can be engineered without substantial additional cost or bulk.

4. Technical improvements in sort rate and efficiency

Key developments in terms of sort rate (presented in terms of X and/or Y live intact sperm s^{-1}) and efficiency of sperm sexing (expressed as output rate divided by input rate) are summarized in Fig. 3. Early work increased efficiency through overcoming sperm orientation losses, thereby increasing the proportion of sperm that could be reliably measured from 20 to 60% and sorted at rates of 100–200 sperm s^{-1} [28]. The introduction of high speed sorters provided the necessary fluidics and data handling capability to improve throughput, and the development of novel orienting techniques significantly increased previous sort rates by an order of magnitude, to reach approximately 1700 s^{-1} [28]. Operation of the purpose-built MoFlo SX in a commercial setting with associated optimization resulted in further sort rate increases to approximately 3000 s^{-1} [29,36]. Further refinements to a number of aspects including sample injection needle precision, nozzle manufacture, and data visualization have reduced measurement variation, thus increasing efficiency and sort throughput to reach 30% and 6000 sperm s^{-1}, respectively [23]. More recently, the availability of sorters with digital electronics has improved sort efficiency with rates now approaching 8000 sperm s^{-1}.

5. Future outlook

Flow sorting has now gained widespread acceptance as an effective and commercially viable means of sexing sperm. In the 20 years since it was first proven to statistically alter sex ratio in live offspring, the technique has been refined to a point where systems can be used for 24 h production with little instrument downtime. Improvements have been made in measurement resolution, sperm orientation, statistics and timing to the point where a single sorter can

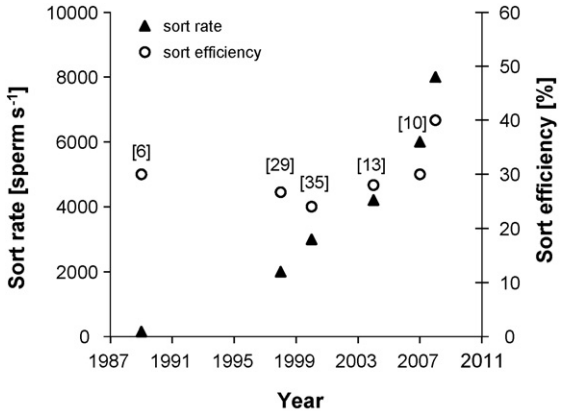

Fig. 3. Time line of improvements in flow cytometer sort rate (▲) and efficiency (o) since the first reported live births using sexed sperm. Sort efficiency (for X or Y sperm) is determined from the ratio of sort rate to sperm analysis rate for a sample with an assumed 50:50 ratio of X:Y sperm (items in brackets indicate references from the literature).

produce sufficient sexed sperm for over 300 straws per day. Inefficiencies in these areas have not been completely eliminated, however, and over 50% of the desired sperm population is still sent directly to waste. With further developments in measurement precision this situation will likely improve, which will benefit the economics of the process, particularly for high value or precious samples. It is also clear that the sorter to sample ratio will continue to increase as sorting facilities are scaled and multi-channel sorters are developed. The current throughput bottleneck may shift from the sorter to other aspects of the overall process such as semen supply from high-demand animals (where suitably qualified bulls may not be able to keep up with demand), or post-sort processing (where the number of straws produced per hour may stretch laboratory resources). There is always the possibility that a new technology, particularly if highly parallel or somewhat less capital-intensive, could very quickly replace the flow cytometer as the sperm sexing instrument of choice. Irrespective of any such developments, there is still significant scope for increased sorter efficiency and throughput through continued advancements of this proven technique.

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