Recent improvements in efficiency of flow cytometric sorting of X and Y- chromosome bearing sperm of domestic animals: a review.

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ABSTRACT

In mammals the only method known to reliably separate X and Y sperm for producing offspring of a specific sex is flow cytometric sorting. This method is based on the observation that X-chromosome bearing sperm of domestic animals contain 3.5-4.2% more DNA than Y-chromosome bearing sperm. Relative DNA content is determined by quantitative staining with Hoechst 33342 and DNA content measured using a modified cell sorter. The major constraint to widespread application of this technology has been the slow sorting rate. Using modified standard speed cell sorters, sample flow rates average 2,000 sperm/sec with 25-35% orientation. Recently, a high speed sorter (MoFlo®) modified for sperm sorting has been used and increases sorting rate by approximately 5-fold. Further, an orienting nozzle has been developed which increases the percentage of sperm that are orientated thus increasing the sort rate by a further 2 to 3-fold. Combined, these improvements have increased the production of sorted sperm from approximately 0.3×10^6/h with conventional sorters to at least 4×10^6/h with the high speed sorter. Preliminary testing suggests that purity of sort and sperm viability are not compromised by increased sorting rates. Pregnancies have been established in pigs and cows with sorted sperm from a modified MoFlo fitted with a novel nozzle. The improvements outlined here greatly enhance the Beltsville Sperm Sexing Technology and make it realistic to consider trials using sex sorted sperm for conventional AI as well as deep uterine AI in cattle.

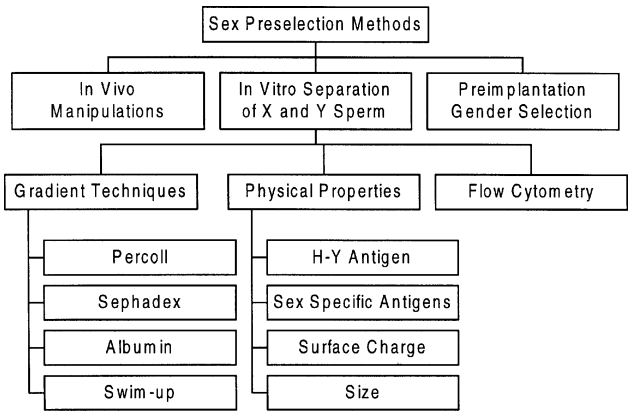
Keywords: X & Y-chromosome bearing sperm; flow cytometry; gender; bovine; pig

INTRODUCTION

The possibility of predetermining the sex of livestock offspring has been a goal of farmers and breeders for many years. The most efficient way to achieve sex preselection is through manipulation of the Y:X ratio of sperm *in vitro* prior to fertilisation. Many methods claiming to separate X and Y sperm have been proposed (see Fig 1) but few have been found to have any merit when examined critically. Most have claimed to exploit some real or theoretical, physical or behavioural differences between X and Y sperm.

Ericsson *et al.* (1973) have proposed that human Y sperm swim faster than X sperm and this forms the basis of a column separation technique using human serum albumin. This technique has been widely used in humans (Dmowski *et al.*, 1979; Beernink *et al.*, 1993) but remains controversial. Recent studies using double labelled fluorescence *in situ* hybridisation (FISH) demonstrate that the technique does not alter the Y:X sperm ratio in sperm samples collected from the column (Vidal *et al.*, 1993; Flaherty *et al.*, 1997).

FIGURE 1: Strategies for predetermination of gender.



For many years H-Y antigen was believed to be localised on the Y sperm only and thus could be used for separation of X sperm. However, Hendriksen *et al.* (1993) showed no differential labelling of H-Y antigen on separate populations of nearly pure X and Y sperm. Investigators continued to search for a surface marker that could be exploited by development of an appropriate antibody and separation technique. Recent evidence would indicate that this is unlikely to succeed. Hendriksen *et al.*, (1996) used sorted X and Y populations to produce protein maps of the sperm surface. No differences were found in surface proteins, leading the authors to conclude that it was unlikely that any sex specific marker existed on the surface of the sperm. A conclusion with which Howes *et al.* (1997) concur following extensive attempts to produce sex specific antibodies using sorted X and Y sperm. Thus for the time being the immunological approach shows little or no promise.

Flow Cytometric Sorting Of Sperm

The basis for sperm separation using a flow cytometer/cell sorter (FCM) is the observation that sperm con-

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taining the X chromosome contain more DNA than those containing the Y chromosome. For domestic livestock this difference ranges from 3.5 - 4.2% (Johnson, 1995). Sperm DNA is quantitatively stained with the fluorescent dye Hoechst 33342 and differences in DNA content can be measured, using a modified FCM (Johnson and Pinkel, 1986).

A major difficulty in analysing and sorting sperm for DNA content is the dense packing of chromatin in the paddle shaped head characteristic of sperm of domestic livestock. Greater fluorescence emits from the edge of the sperm head when sperm are stained with Hoechst 33342 and excited by a UV laser beam. In order to adequately resolve the small differences in DNA content between X and Y-chromosome bearing sperm, it is necessary to modify commercial cell sorters so that collection of the fluorescence signal can be controlled according to the orientation of the sperm head to the excitation source (Johnson and Pinkel, 1986).

Briefly, the process has involved taking aliquots of 15 x 10^6 sperm/ml and diluting with a buffer appropriate for the species. Hoechst 33342 is then added to give a final concentration of ♠ 7.2 µM and the sample incubated at 32-35°C for - 1h. Sperm are sorted into tubes containing a small amount of TEST-yolk buffer (Johnson *et al*., 1989) which enhances post-sort sperm survival.

The performance of a modified FACStar Plus (Becton Dickinson, San Jose, CA, USA) is shown in Table 1, column 1. Sperm sorted using similarly modified standard speed cell sorters have been used in in vitro production (IVP) systems to produce modest numbers of bovine (Johnson *et al*., 1994; Cran *et al*., 1995) or porcine (Rath *et al*., 1997) embryos, or for surgical insemination of pigs (Johnson, 1991) and sheep (Cran *et al*., 1997).

TABLE 1: Typical sort parameters for a modified standard speed cell sorter and a high speed cell sorter modified for sperm sorting with and without a novel nozzle fitted (Rens et el., 1997).

	FACStar Plus[1]	MoFlo[2]	MoFlo[3] + novel nozzle
Sample pressure (psi)	♠ 13	♠ 60	♠ 60
Max flow rate (/sec)	6,000	30,000	30,000
Sample flow rate (/sec)	2,500	15,000	15,000
Sort efficiency single sex (%)	♠ 3.0	♠ 3.0	♠ 7.5
Orientation (%)	25-35	25-35	65-75
Sort Purity (%)	♠ 90	♠ 90	♠ 90
Sort rate single sex (x 10^6/h)	0.15-0.30	1.0-2.0	4.0-5.0

[1] Beaumont and Gurnsey, unpublished
[2] Johnson and Welch, unpublished
[3] Welch *et al*., 1998

Recent Improvements In Flow Cytometric Sorting Of Sperm

Numerous changes to sperm processing protocols for sorting sperm into X and Y populations have occurred over the past several years. These changes have resulted in improved consistency in the purities of sorted X and Y sperm using standard sperm sorting instrumentation (Johnson, 1997).

However, two significant improvements have been reported in the past year that have moved the sperm sexing technology to a new level of application. Firstly, sperm orientation was improved two to three fold over that attained with the original bevelled needle (Rens *et al*., 1997). The improvement resulted from the redesign of the nozzle which improved the percentage of sperm oriented from 30 ± 5% to 70 ± 5%.

Secondly, the Beltsville group modified a high speed cell sorter for sorting sperm (MoFlo, Cytomation, Inc, Ft. Collins, CO, USA; L.A. Johnson and G.R. Welch, unpublished) The high speed sorter resulted in improved sorting rates (see Table 1, column 2), approximately five times those achieved with standard speed sorters (0.3 x 10^6 vs. 1.5 x 10^6/h). The most recent report from this group (Welch *et al*., 1998) describes the adaptation of the novel orienting nozzle (Rens *et al*., 1997) to the high speed MoFlo sperm sorter (Table 1, column 3). The resulting sperm production/h is improved to 4 - 5 x 10^6 X and Y sperm. This represents a 10-12 fold improvement in sperm production from rates achieved with standard speed sperm sorters.

The MoFlo high speed sorting system adapted for sperm as described, has been used in several trials to test the efficacy of the sorted sperm. Two trials have been conducted with boar sperm, using this modified system. All the sorted sperm were sorted at Beltsville and used for IVF of *in vitro* matured (IVM) oocytes at Beltsville (Long *et al*., 1998) or shipped from Beltsville to Columbia, MO for IVF of IVM oocytes in that laboratory (Day *et al*., 1998). In both studies the results showed a production of litters with 97% females (Day *et al*., 1998; Johnson *et al*., 1998a). The key finding however, was the normal fertilisation and production of offspring from sperm that had been subjected to high pressure associated with high speed sorters (40-50 psi).

The increased production rate of sperm with the high speed system was also used to obtain pregnancies in cattle using 5 x 10^6 sperm/conventional insemination (Johnson *et al*., 1998b) and in cattle using deep uterine insemination with approximately 0.3 x 10^6 sperm/insemination (Seidel *et al*., 1998). Earlier, Seidel *et al*. (1997) had successfully established pregnancies using sperm sorted with a modified standard speed sorter using deep uterine insemination.

TABLE 2: Potential number of cow oocytes fertilised and recipient cows inseminated per hour of sorting using standard and high speed cell sorters.

	Standard speed FCM *	MoFlo	MoFlo + novel nozzle
No. of X or Y sperm (x 10^6/h)	0.15-0.30	1.0-2.0	4.0-5.0
IVP 10,000 sperm/oocyte	15-30	100-200	400-500
2,000 sperm/oocyte	75-150	500-1000	2000-2500
Deep uterine AI (300,000/cow)	- 1	3-6	12-16
Conventional AI (1 x 10^6/cow)	< 0.3	1-2	4-5

* FACStar Plus, Becton Dickinson, San Jose, CA, USA
* Epics V 750 series, Coulter Corporation, Miami, FL, USA

The impact of improvements in the technology on its possible application in the cattle breeding industry can readily be seen in Table 2. It is clear that the application of standard speed cell sorters would restrict the use of sorted sperm to IVP systems. If abattoir derived oocytes were used in such a system it is possible that the supply of sorted sperm might become limiting. However, with the introduction of high speed sorters provision of sorted sperm is unlikely to be a problem, rather, the supply of suitable oocytes may become limiting. Although the latest improvements in the sorting technology and sperm processing steps does not yet make the use of sorted sperm in conventional AI commercially practical, they would provide sufficient sorted sperm for use in deep uterine AI in cattle.

CONCLUSIONS

Flow cytometric sorting of sperm is the only method which has been demonstrated to substantially skew the sex ratio reliably and repeatably in domestic livestock. However, until recently this technique has had limited application largely because of constraints on the number of sperm sorted. The rate at which sperm may be sorted is limited by 3 factors: sample flow rate, sperm orientation and resolution of DNA differences. The first of these factors has been addressed with the recent availability of high speed cell sorters. Sperm orientation has been greatly increased with the development of a novel nozzle. The combined effect of these developments has been to increase the rate of sorting by 10 to 12-fold so that 3-5 x 10^6 X and Y sperm may be sorted/h without compromising purity of sort or sperm viability. One can easily achieve greater than 90% of the desired sex of offspring in cattle and swine and other animals using the improved sperm sorting technology reviewed here. Sufficient quantities of sorted sperm can now be made available for cattle trials using deep uterine AI or conventional AI using fresh sorted semen. Research on the freezing of sorted sperm has indicated reduced survival compared to controls (L.A. Johnson and D.G. Cran, unpublished). With more research this problem should be solved, leading to wider application of the Beltsville Sperm Sexing Technology.

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