# Application of sexed sperm

2011 11/ 21 Feng Hsiang chu

Physiological department

# Outline

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   2.1. Application of the HMG box of bovine SRY gene for sex determination
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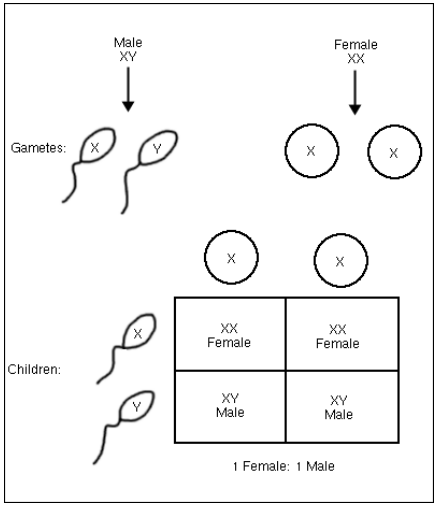
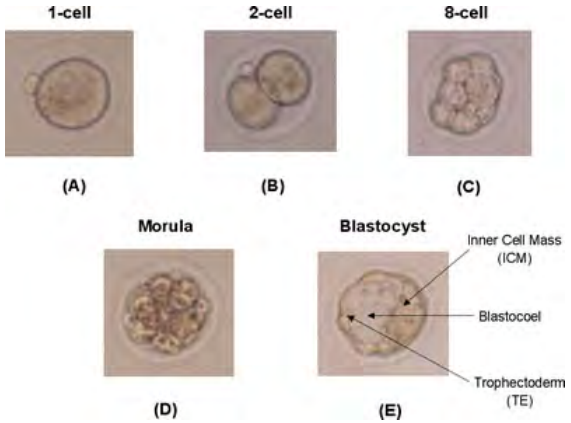
4. Overview of sexing sperm
   4.1. AI with cryopreserved sexed sperm
   4.2. IVF of bovine embryos using sex-sorted sperm
5. Conclusions

# Introduction

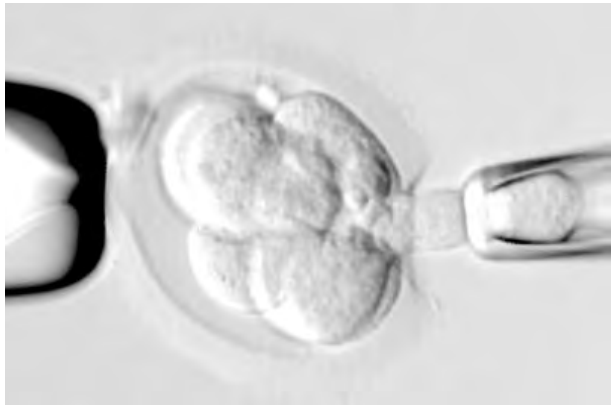
- For thousands of years, livestock owners have desired a methodology to predetermine the sex of offspring for their herds
- 1 Holstein heifer calves selling for $ 510–590, 1 Holstein bull calves sold for $ 170–208
- different techniques, such as the Quinacrine mustard staining for Y-chromosome, the Quantitative Southern Blotting, the semi-quantitative PCR, the multicolour fluorescence in situ hybridisation (FISH) have been developed



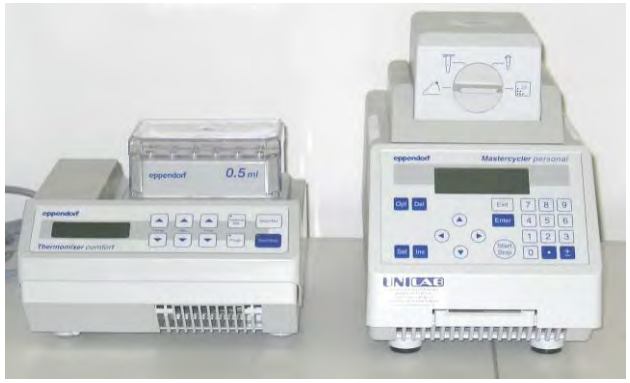
# Sexing preimplantation embryos by PCR



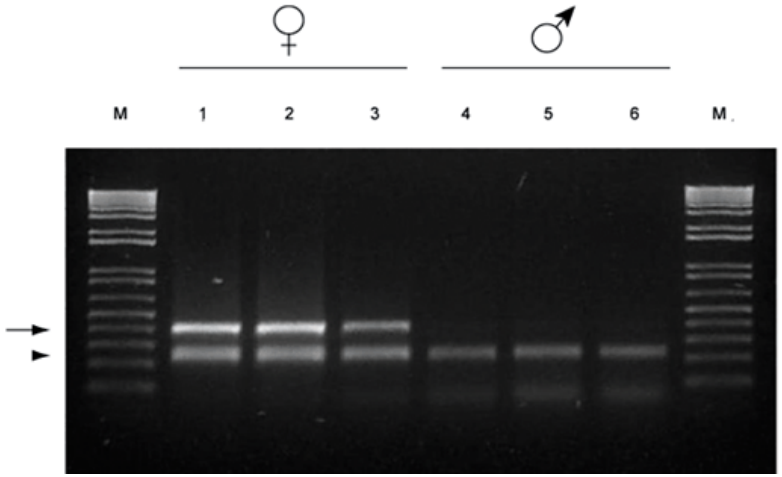

Embryos in vitro



micromanipulation



PCR mechanism




Gel electrophoresis

# Application of the HMG box of bovine SRY gene for sex determination

- Amplification of the bovine high motility group (HMG) box of the sex-determining region of the Y chromosome gene (SRY).
- The open reading frame (ORF) of human SRY gene is contained within a single exon and encodes a 204-amino-acid protein.
- The central 79 amino acids encode the HMG box, which functions as a DNA-binding and DNA-bending domain and also contains 2 nuclear localization signals.
- Comparison of the amino acid sequence of the HMG box of the SRY gene among human, mouse, rabbit, wallaby, marsupial mouse, and sheep revealed 70% identity.
- There is no sequence conservation outside the HMG box.

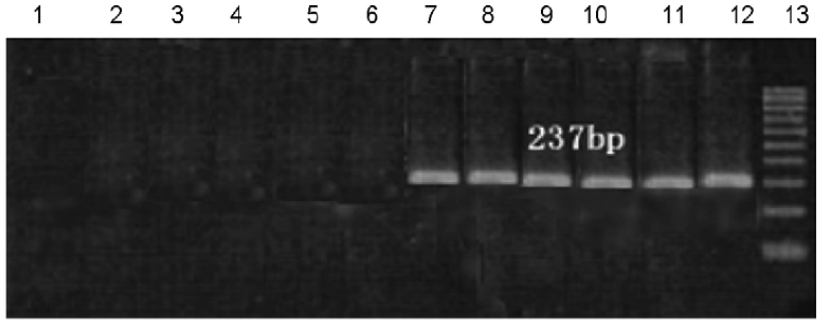



Fig. 1. Agarose gel electrophoresis of PCR products of genomic DNA extracted from male and female bovine, using HMG primers. Lanes 1–6 are PCR products from reactions using genomic DNA prepared from the testes of six female bovines. Lanes 7–12 are PCR products from reactions using genomic DNA prepared from six male bovines. Lane 13 is 1000 bp PCR low ladder (Sigma). The position of the 237 bp is male-specific PCR product.

Table 1
Accuracy of sexing bovine embryos

| | |
|---|---|
| Embryos | |
| Sexed | 14 |
| Transferred | 14 |
| Calves | |
| Born (%) | 9/14 (64.3) |
| Accuracy (%) | 9/9 (100) |



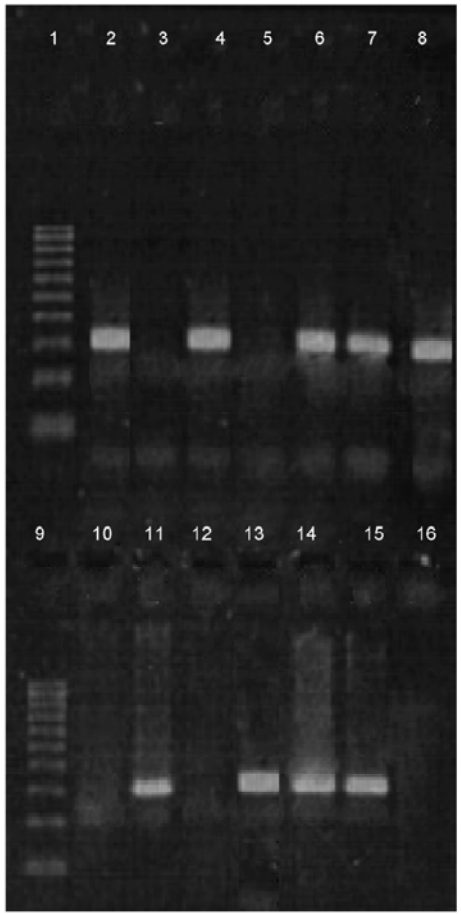
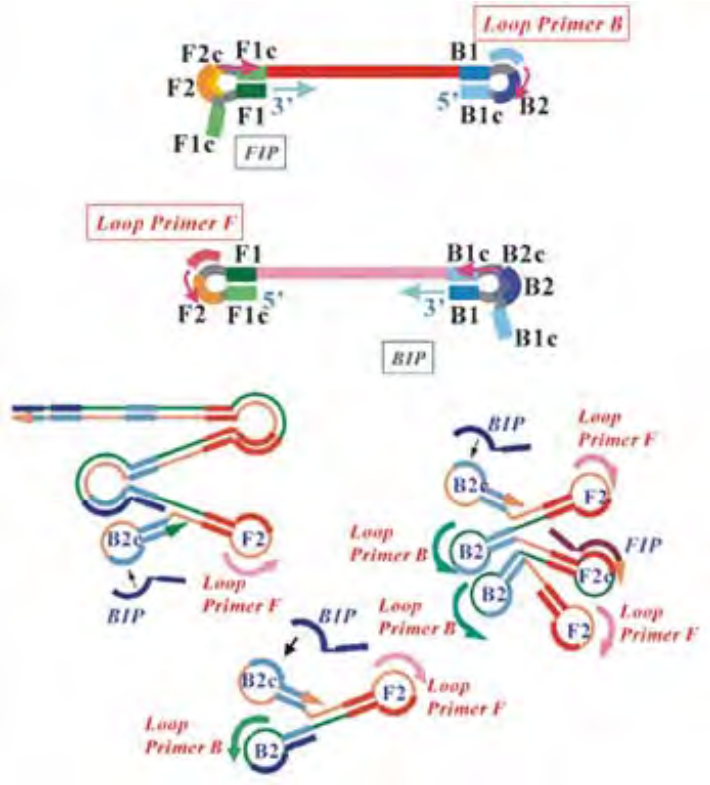
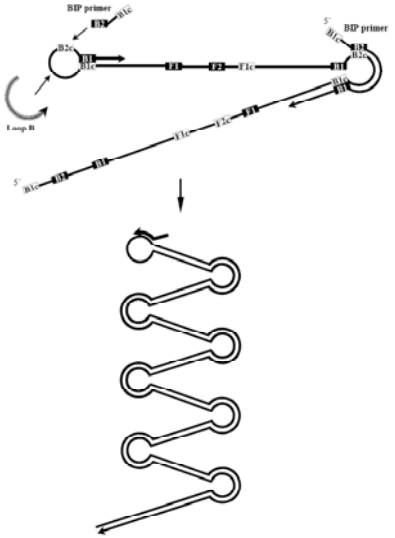



Fig. 2. Sexed embryonic cells using designed male-bovine specific primers by PCR. Lanes 1 and 9 are 1000 bp PCR low ladders (Sigma); Lanes 3, 5, 10, 12 and 13 are female embryos; Lanes 2, 4, 6, 7, 8, 11, 13, 14 and 15 are male embryos.

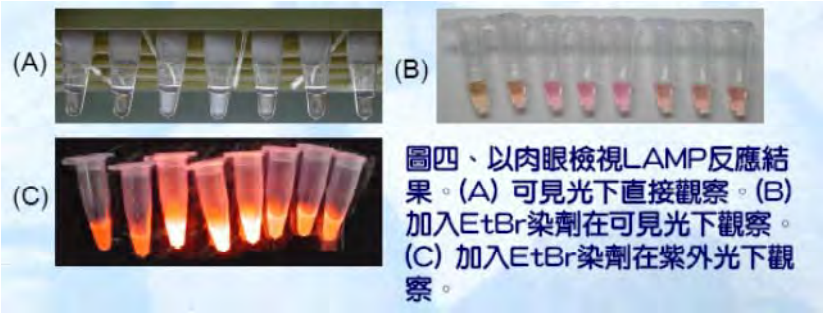
*W.Lu et al 2007*

# Using loop-mediated isothermal amplification



The Loop Primers (either Loop Primer B or Loop Primer F), containing sequences complementary to the single stranded loop region (either between the B1 and B2 regions, or between the F1 and F2 regions) on the 5' end of the dumbbell-like structure, provide an increased number of starting points for DNA synthesis for the LAMP method. An example is shown in the figure where there is an amplified product containing six loops. In the original LAMP method, four of these loops would not be used, but through the use of Loop Primers, all the single stranded loops can be used as starting points for DNA synthesis.



# sexing of bovine preimplantation embryos using loop-mediated isothermal amplification

Primer sequences for LAMP-based embryo sexing

| | |
|---|---|
| Male-specific primers | |
| Inner primer F | 5′-AGCTATGTGGCATGTGGATCCTTCCCTGGAAATGTTTAAGTG-3′ |
| Inner primer B | 5′-TAAAGCCAGACACAGAGGTCACTTTTGCTTCTCTTTCCTGCTTC-3′ |
| Outer primer F | 5′-AGCCAAGAAGTGGATGAATC-3′ |
| Outer primer B | 5′-GCAGTGCATTTCCTCCTC-3′ |
| Loop primer F | 5′-GGGATGAAACTGTGCAT-3′ |
| Loop primer B | 5′-ATTGCATGTGGAAGAACTGTAG-3′ |
| Male–female common primers | |
| Inner primer F | 5′-GAGGAACATTGGCTTCTGGACAAGCTGGGGATTGCTCT-3′ |
| Inner primer B | 5′-AGTGGAAGCAAAGAACCCCACCCAGTGAGCTCCAA-3′ |
| Outer primer F | 5′-AGGCTGCCTCTTGTGTT-3′ |
| Outer primer B | 5′-CATGGCCTAGAGACCAATC-3′ |
| Loop primer F | 5′-CCTAGATGAGGTCTATTGGC-3′ |
| Loop primer B | 5′-CTGCTCTCGAATTGTGACG-3′ |

cycles of shuttle PCR at 98 °C for 8 s and at 66 °C for 20 s. The final extension step was followed by 5-min incubation at 72 °C.


Effect of cell number on sensitivity and accuracy of LAMP-based embryo sexing

| Number of blastomeres used for assay | Number of embryos examined | Number (%) with satellite sequence detected | Number (%) correctly determined |
|---|---|---|---|
| 1 | 15 | 12 (80.0) | 9 (75.0) |
| 2 | 28 | 26 (92.9) | 23 (88.5) |
| 3 | 16 | 13 (81.3) | 13 (100) |
| 4 | 16 | 16 (100) | 16 (100) |
| 5 | 17 | 17 (100) | 17 (100) |

DNA of blastomeres was extracted with treatment at 95 °C for 5 min (heat method).

*H. Hirayama et al 2004*

# Sexing of embryos by developmental arrest induced by H -Y antisera

- embryos at the late morula stage were cultured in medium containing high-titer rat H-Y antisera
- After 12 h of incubation, embryos blocked at the late morula stage were classified as males and those at the blastocyst stage were classified as females.
- that 83% of the embryos classified as males and 82% of those classified as females had their sex correctly predicted
- was an efficient strategy for non-invasive embryo sexing

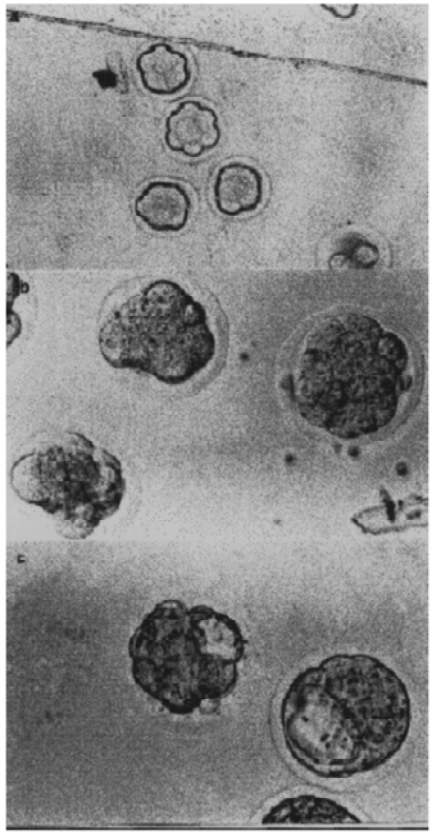


Fig. 1. (a) Morulae and compact morulae before treatment with high-titer rat H-Y antisera; (b) embryos at the compact morula stage after being cultured for 12–24 h in the presence of high-titer rat H-Y antisera (classified as males); and (c) embryos at the blastocyst stage after being cultured for 12–24 h in the presence of rat H-Y antisera (classified as females).

Sexing murine embryos by inducing developmental arrest with high-titer rat H-Y antisera

| Blastocoele formation (presumptive sex) | No. (%) embryos | No. (%) embryos sexed by chromosomal analysis | |
|---|---|---|---|
| | | Male | Female |
| No (male) | 60 (51.3) | 50 (83.3) | 10 (16.7) |
| Yes (female) | 57 (48.7) | 9 (15.8) | 48 (84.2) |
| Total | 117 | 59 | 58 |

The genetic sex was confirmed by chromosomal analysis.

*M.F.P.D.-T. Ramalho et al 2004*

# Overview of sexing sperm

**8,000-6,000 B.C.**

**Humans Begin Herding Animals Mesopotamia – sheep Egypt – cattle & goats China – poniesMid-East – camels South America - llamas**

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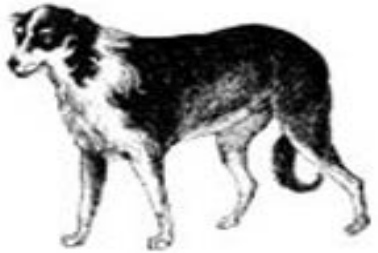
**1780**

**First well-documented artificial insemination using dogs.**



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**1910**
**Artificial Insemination**
**Humans place bull semen into a cow's reproductive tract. Today, more than 65% of U.S. dairy herds, 85% of U.K. dairy herds and 90% of Scandinavian dairy herds are bred by A.I.**

⇩

**1950s**
**Frozen Sperm**
**Sperm that is deep-frozen then thawed is shown to produce healthy offspring.**
**Today, 99% of all A.I.'s in the U.S. and U.K. dairy industries use frozen/thawed semen.**

⇩

**1970s**

**Flow Cytometer**
**Equipment first developed to sort living cells at high speed.**

⇩

**1992**

**First Sex-Selected Calf**
**Mastercalf, Ltd., of Cambridge, U.K., produces world's first sex-selected calf by in-vitro fertilization.**

**Mid-1990s**

**Separation of X- and Y-Bearing Sperm**
Further advances in flow cytometry and low-dose insemination permit Colorado State University researchers to produce the world's first sex-selected calf by artificial insemination.

**1997**

**XY Inc. Acquires Mastercalf of U.K.**
**and achieves world control of the sexing technology of animals. XY Inc. also produces its first sex-selected calf by artificial insemination.**

**2005**

**World's First Sex-Selected Dolphin.** In October 2005 the world's first sex-selected marine mammal, an Atlantic bottlenose dolphin, is born at SeaWorld San Diego via XY® Inc. sex-selection technology.

**2006**
**World's First Sex-Selected Kittens**
In October 2006, the world's first sex-selected domestic cats were born with their sex predetermined. The litter, produced from embryos fertilized with sexed sperm, was born at Audubon Center for Research of Endangered Species in New Orleans.

**2007**
**World's First Sex-Selected Dogs**
In January 2007, the world's first sex-selected dogs were born, demonstrating XY® Sex Selection Technology works in the canine world.

# separated X- and Y-sperm by sperm sorter

- The ability to sort individual sperm cells into viable X- and Y-chromosome-bearing fractions made producers' sex selection dream a reality in the 1990s
- Semen can be sexed with greater than 90% accuracy with use of a flow cytometric cell sorter
- There are, however, slight differences in the sexing accuracy between X-sorted sperm (87.8%) and Y-sorted sperm (92.1%) in calves born
- Semen sexing, involving the separation of X- from Y-chromosome bearing sperms, implies its application in artificial insemination (AI) or in *in vitro* fertilisation (IVF) with the subsequent embryo transfer (ET).

# How sperm are sexed

# 精子分離的原理

- the X-sperm contains more DNA than the Y-sperm (approximately 4% more in the case of cattle)

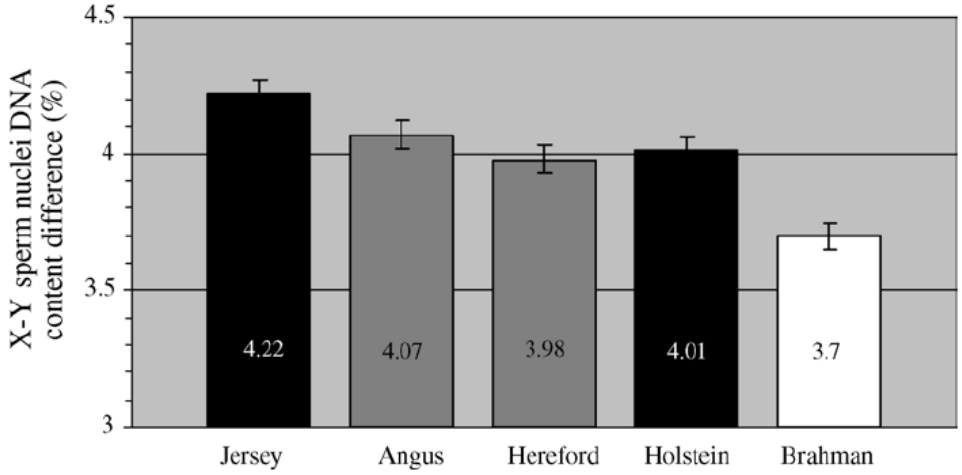
  X-精子比Y-精子染色體多4 %

- X-sperm bind more dye than Y-sperm, they give off 4% more fluorescence, which the computer can recognize  flow cytometric cell sorter

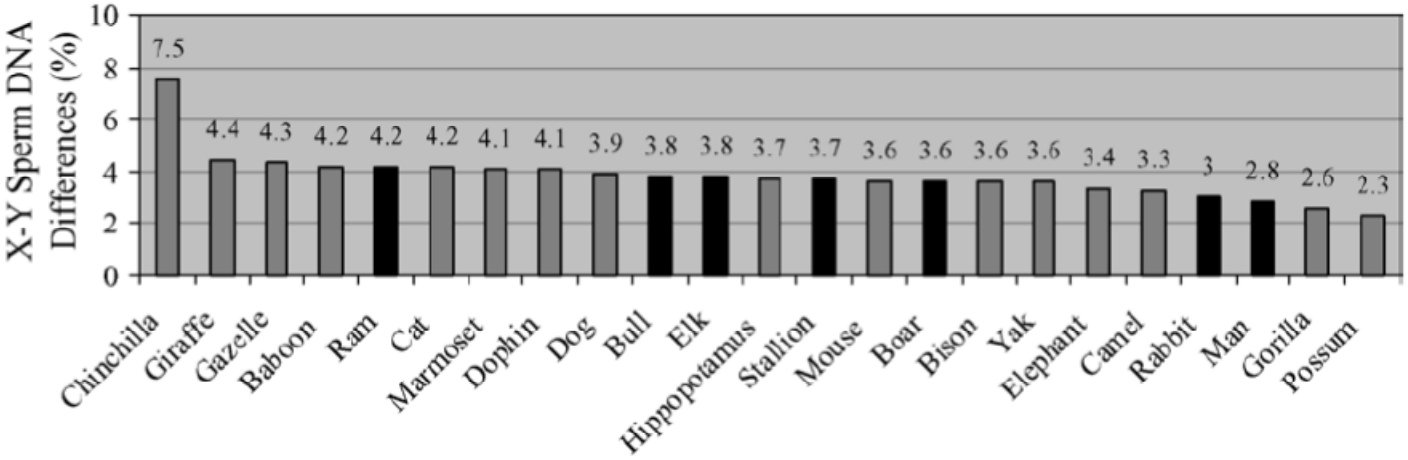
  利用螢光染色與雷射判讀其差異

- this technology is characterized by high costs, complexity of implementation and lower pregnancy rates than with control sperm.

  分離過程會對精子造成一定程度的傷害




不同品種牛隻其X-Y精子染色體量的差異也不同




*D.L. Grarner 2006*

# 精子的型態越扁平對性別分離越有利

***Sambar deer***

***8.4***

**(red deer: 8.0)**

***6.0*** **(4.5)**

***37.8*** **(30.6)**

**X-Y difference 3.8**

**143.6**

Dimensions and profiles of sperm heads and flow cytometric sorting indices for some domestic mammals and man[a]

| Dimension | Bull | Boar | Ram | Rabbit | Cat | Dog | Horse | Man |
|---|---|---|---|---|---|---|---|---|
| Length (μm) | 9.1 | 9.0 | 8.1 | 7.7 | 7.7 | 7.0 | 6.5 | 4.6 |
| Head sagittal section |  | | | | | | | |
| Width (μm) | 4.7 | 5.0 | 4.0 | 4.5 | 3.2 | 3.5 | 3.4 | 3.2 |
| Head profile |  | | | | | | | |
| Area ($\mu m^2$) | 34.5 | 37.5 | 26.6 | 28.0 | 19.0 | 20.9 | 15.2 | 10.8 |
| X-Y difference (%) | 3.8 | 3.6 | 4.2 | 3.0 | 4.2 | 3.9 | 3.9 | 2.8 |
| Sorting index[b] | 131 | 115 | 112 | 84 | 80 | 82 | 59 | 31 |

[a] Compiled from Mann [67] Mann and Lutwak-Mann [68], Johnson [7], Welch and Johnson [6], Garner [11], Garner and Seidel [13] and Seidel and Garner [14].
[b] An approximation of the ability to flow cytometric sort sperm consisting of the head profile area ($\mu m^2$) × X–Y Sperm DNA difference (%).



*D.L. Grarner 2006*

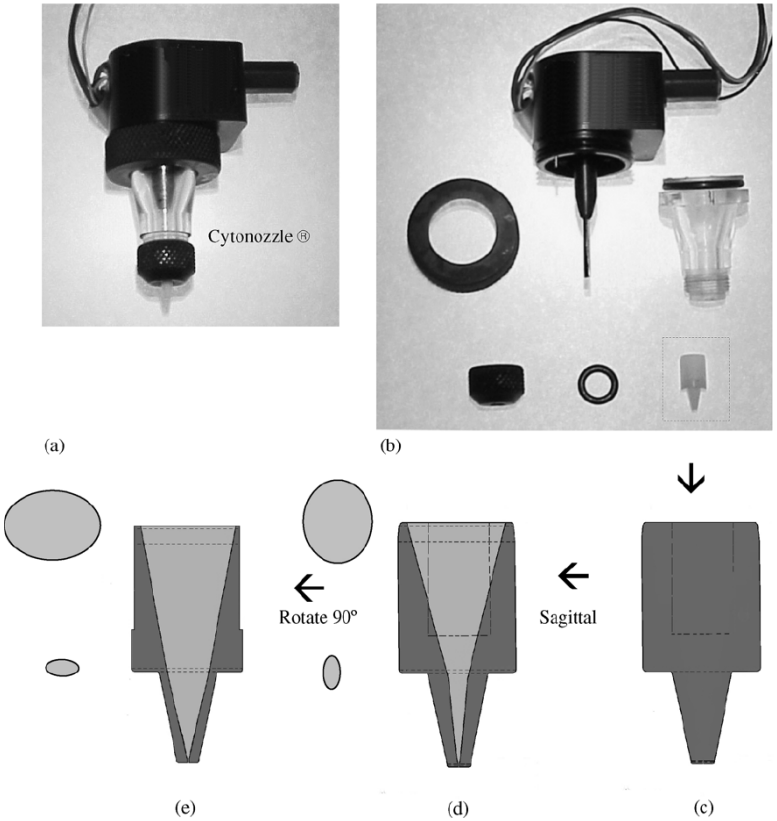
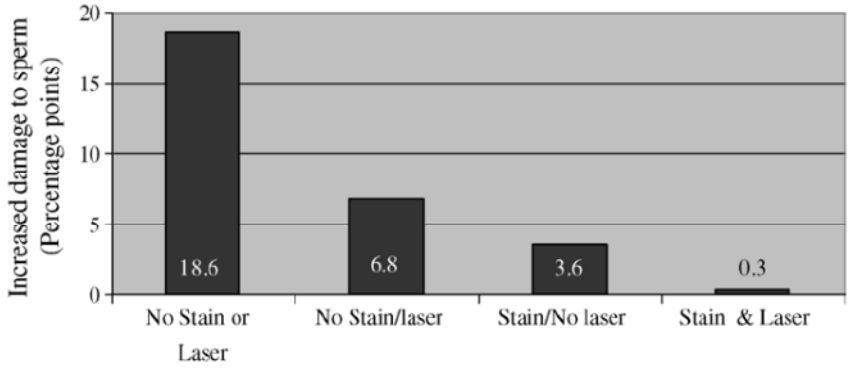



Fig. 3. Illustration of an assembled Cytonozzle® (a); a disassembled nozzle showing the flow chamber, tapered injection needle and the ceramic tip [surrounded by dotted lines] (b); a profile of the ceramic tip (c); sagittal section of the tip showing the narrowest elliptical orienting configuration [shaded cross sections of the tip interior are shown on the immediate left of the tip] (d); and a sagittal section after rotation of the tip 90° the illustrate the widest portion of the elliptical internal bore of the tip [shaded cross sections of the tip interior are shown on the immediate left of the tip] (e).



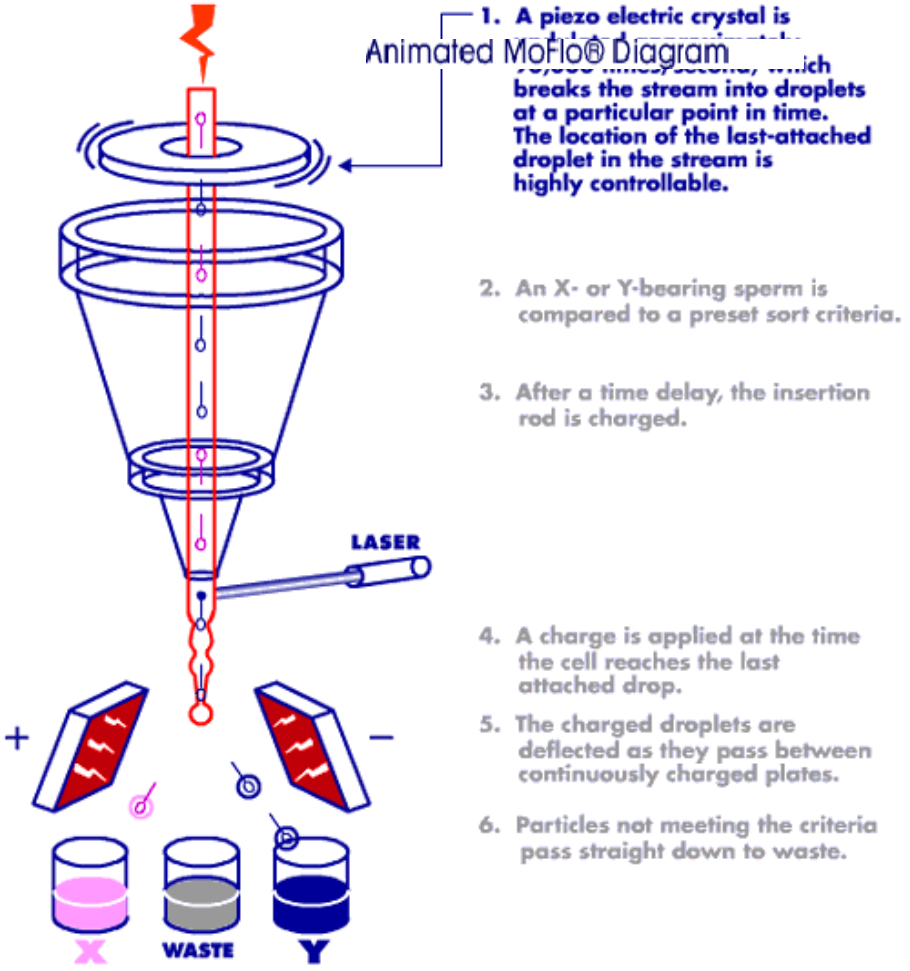

分離過程造成的傷害主要來自

1.雷射光照射

2.DNA螢光染色

3.稀釋與等待時間

*D.L. Grarner 2006*




分離精子的速度從1990年到現今一直在提升

35萬隻/小時~2000萬隻/小時

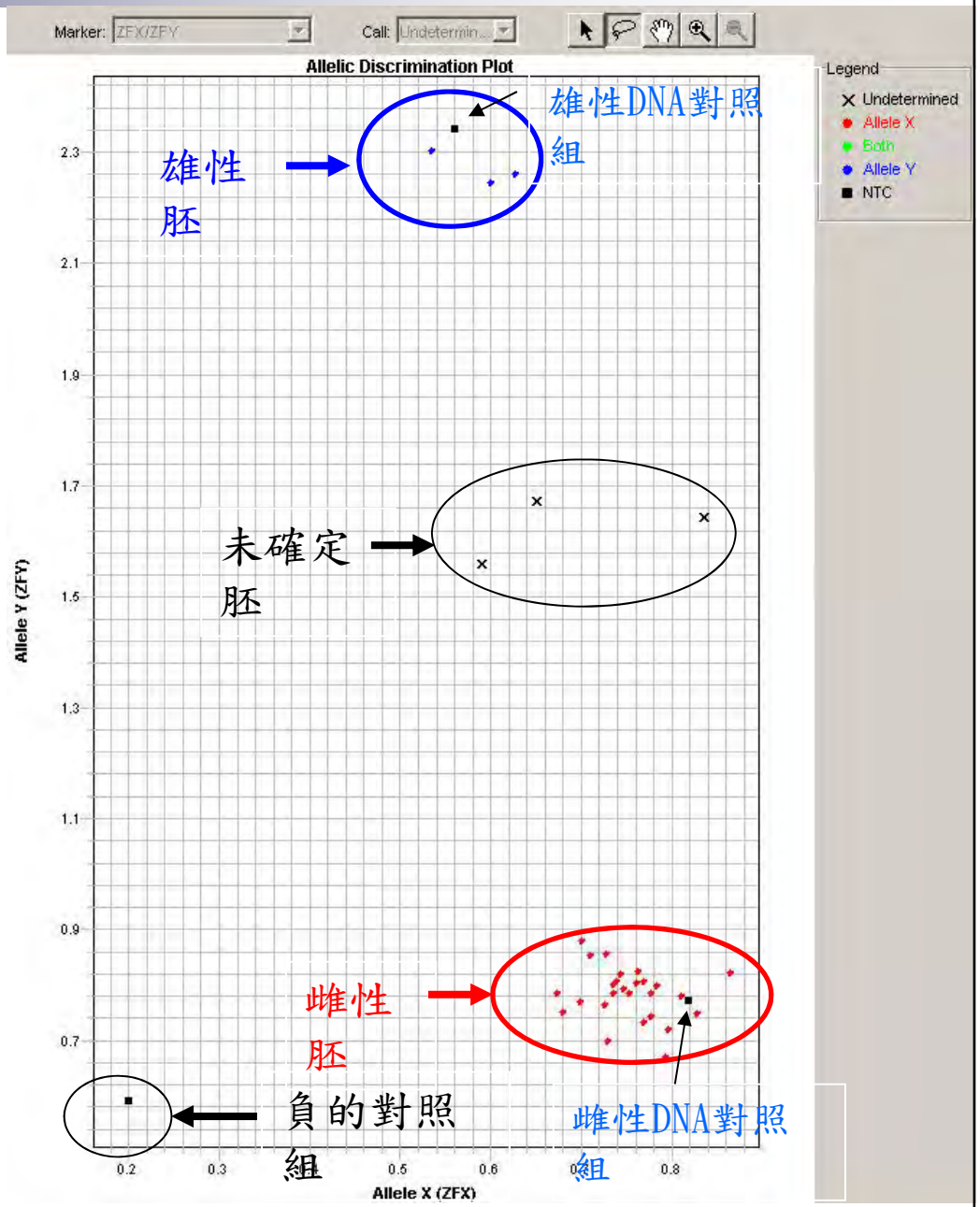
In practice, about 20% of sperm end up in the X-fraction, 20% in the Y-fraction and 60% are damaged or not sexable

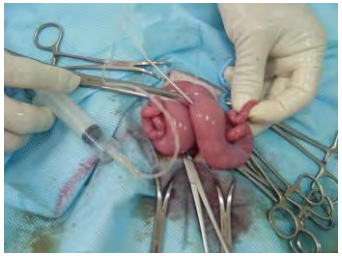
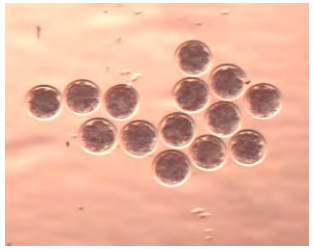
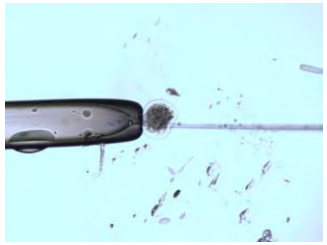
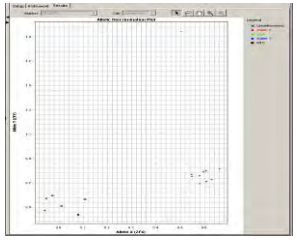
# BECKMAN com **MoFlo XDP's**

- **Drop formation frequency: 200 kHz**
  - **每秒可震出 200,000 顆水珠**
  - **為維持分選純度，平均每 3 顆水滴含有 1 顆細胞為最佳狀態**
- **Sort speed up to 70,000 eps**
  - **Purity > 99%；Yield > 90%**
- **IntelliSort: 自動化協助設定分選條件，並監控分選過程**
- **4Way sorting**
- **3 different sort modes and mixed mode**
  - **Enrich mode**
  - **Purity mode**
  - **Single mode**
- **CyClone deposition system**

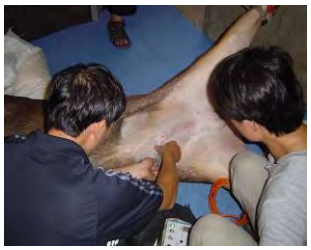
BD com **influx**

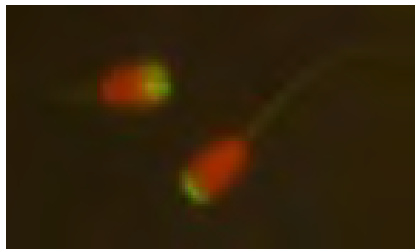
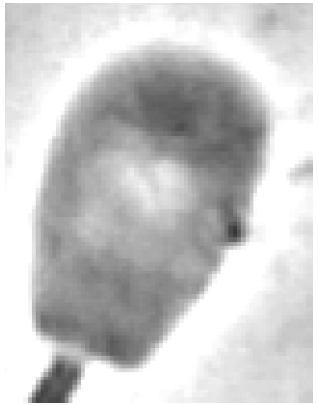
- 乳牛選性冷凍精液受精後，發育至8-16細胞期33個牛胚利用 Real time PCR分析結果。雌性胚比率為90% 。
- After the cow chooses the frozen sperm fertilization, grows uses Real time to 8-16 cell time 33 cow embryos the PCR analysis result. The female embrionic ratio is 90%



- 水鹿精子型態及分離條件之評估冷藏保存技術建立（高雄場）

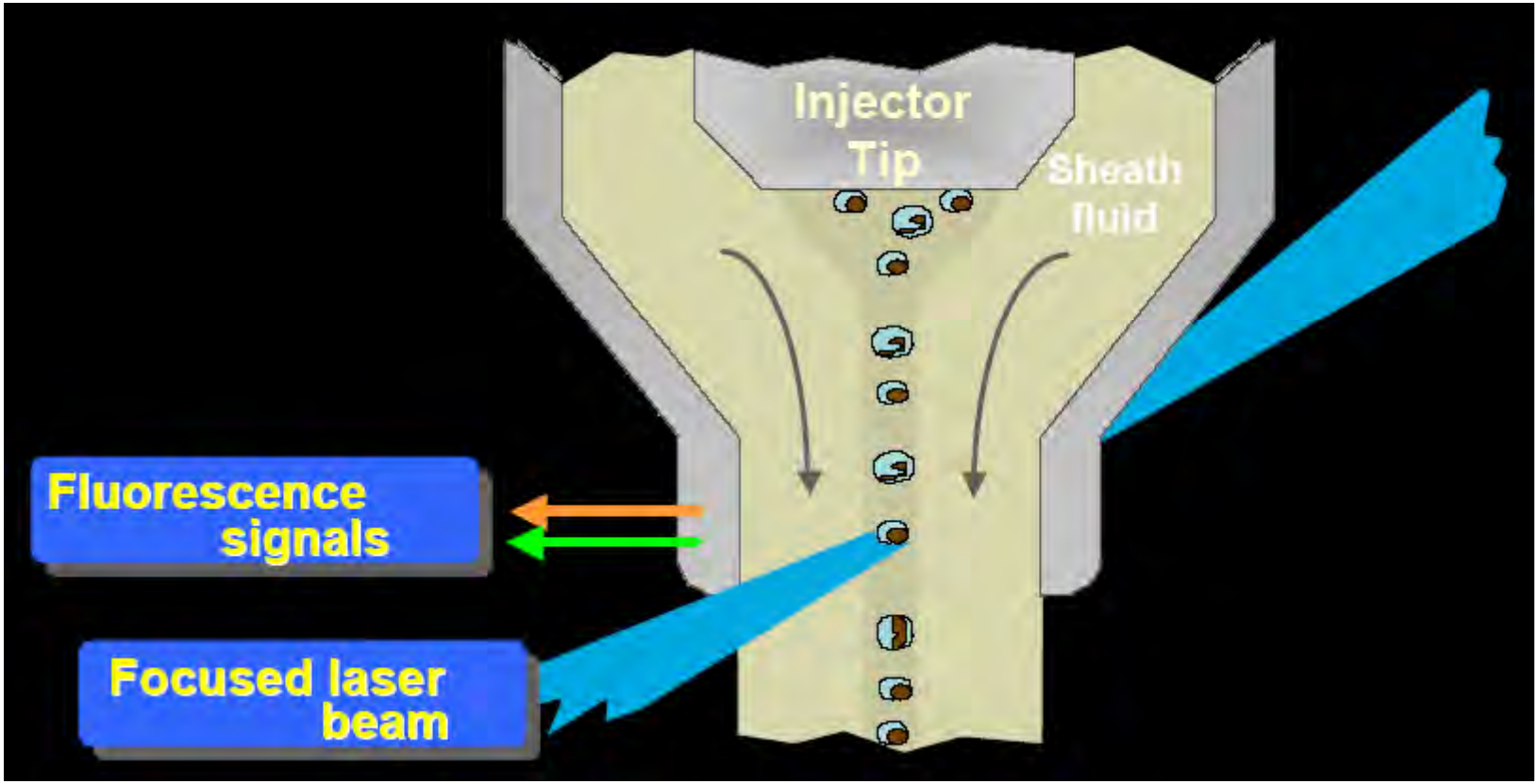


# Fluid System: Hydrodynamic Focusing

讓細胞在管路中排路隊

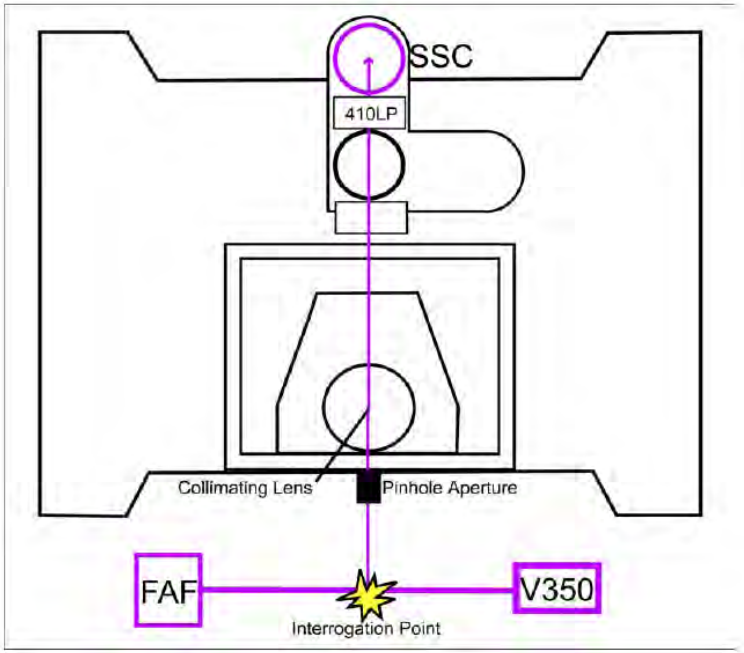
***Hydrodynamic Focusing***（流體動力聚焦）



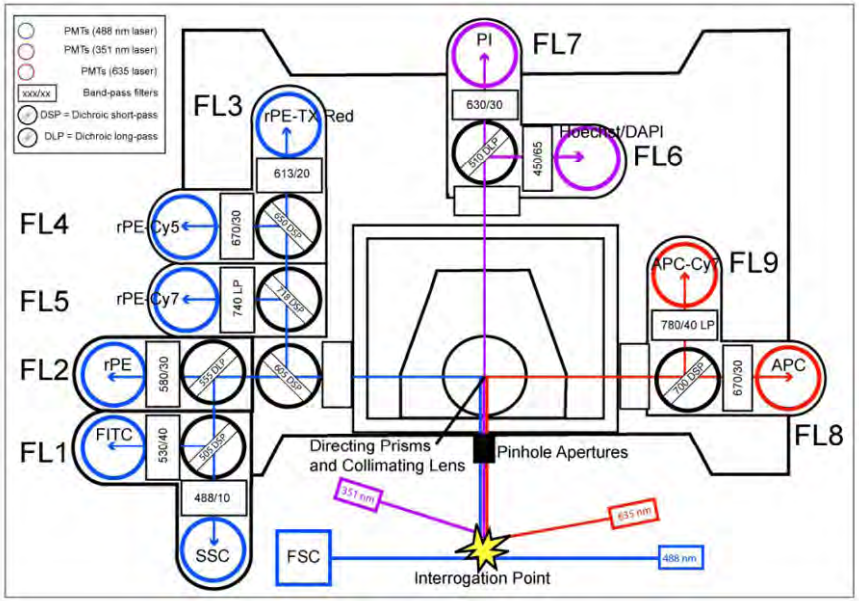
# Sperm sorting V.S. cell Sorter差異

1. Optical System diferent

Sperm sorting




Sorter



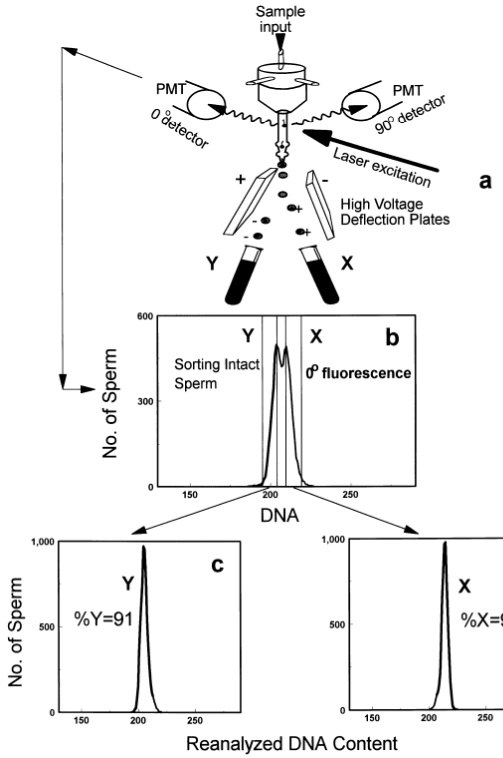

Wave length 350

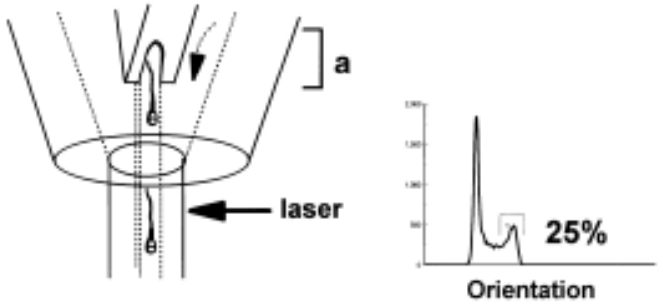
# 2. Sorting Nozzle System diferent

**The Beltsville Sperm Sexing Technology: High-Speed Sperm Sorting Gives Improved Sperm Output for In Vitro Fertilization and AI**

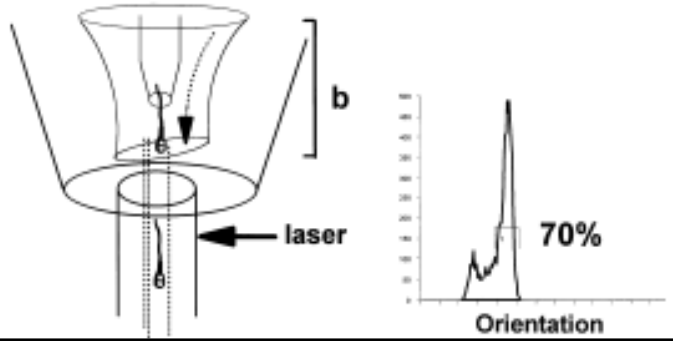
Lawrence A. Johnson, Glenn R. Welch and Wim Rens

*J Anim Sci* 1999. 77:213-220.




**New Orienting Nozzle**



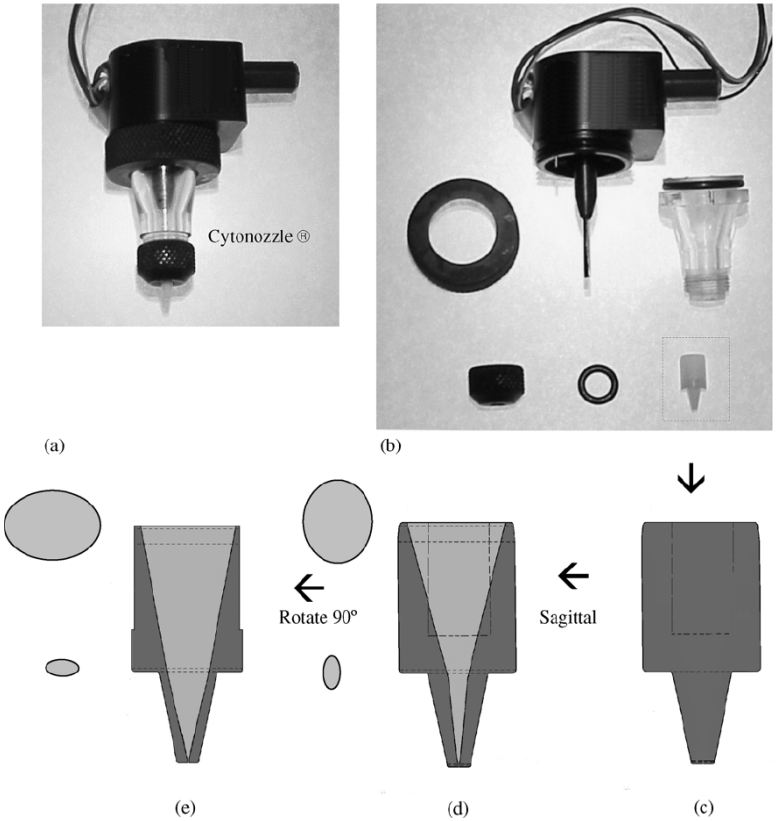
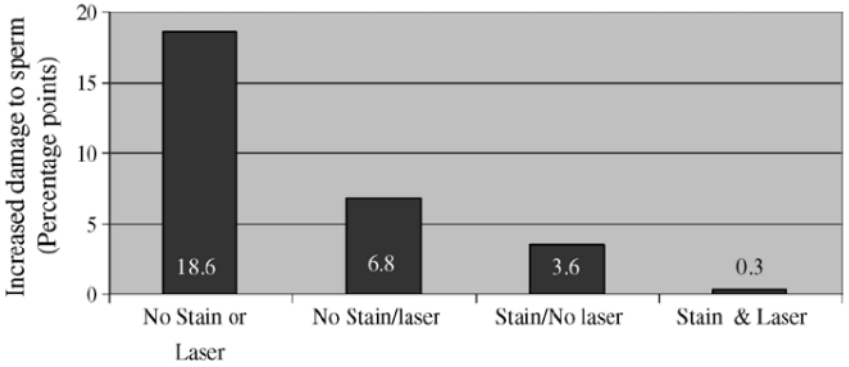


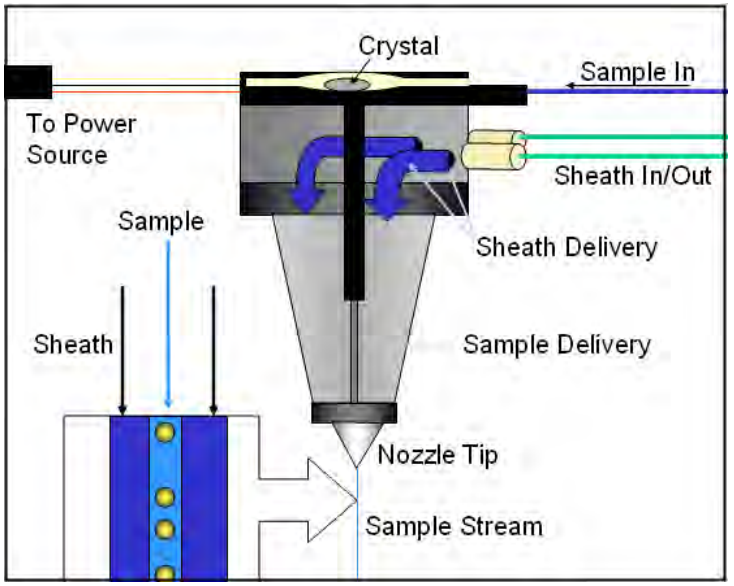

Fig. 3. Illustration of an assembled Cytonozzle® (a); a disassembled nozzle showing the flow chamber, tapered injection needle and the ceramic tip [surrounded by dotted lines] (b); a profile of the ceramic tip (c); sagittal section of the tip showing the narrowest elliptical orienting configuration [shaded cross sections of the tip interior are shown on the immediate left of the tip] (d); and a sagittal section after rotation of the tip 90° the illustrate the widest portion of the elliptical internal bore of the tip [shaded cross sections of the tip interior are shown on the immediate left of the tip] (e).

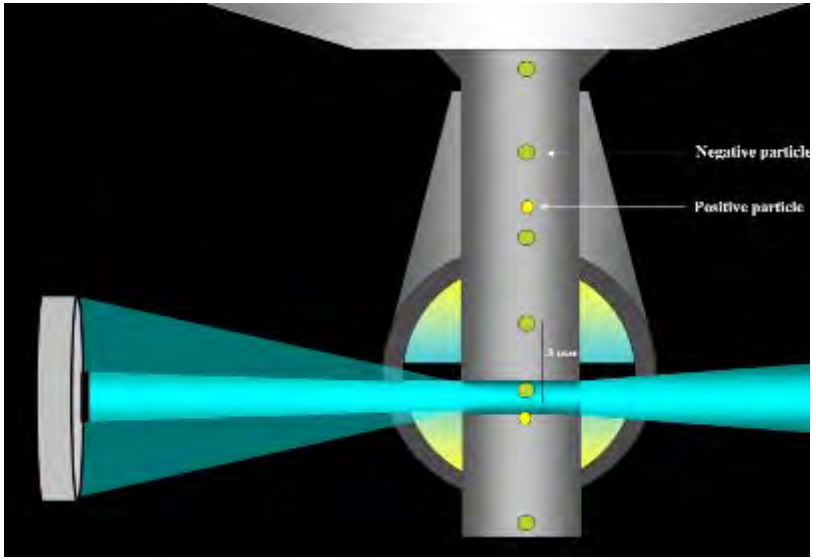



D.L. Grarner 2006

# Fluidic Design

- **Jet-in-Air stream design**
- **Faster velocity of stream to achieve high speed analysis and sorting (Sheath pressure: 4~100 psi)**
- **The Faster velocity generated very short signal pulse to improve the cell distinguished**



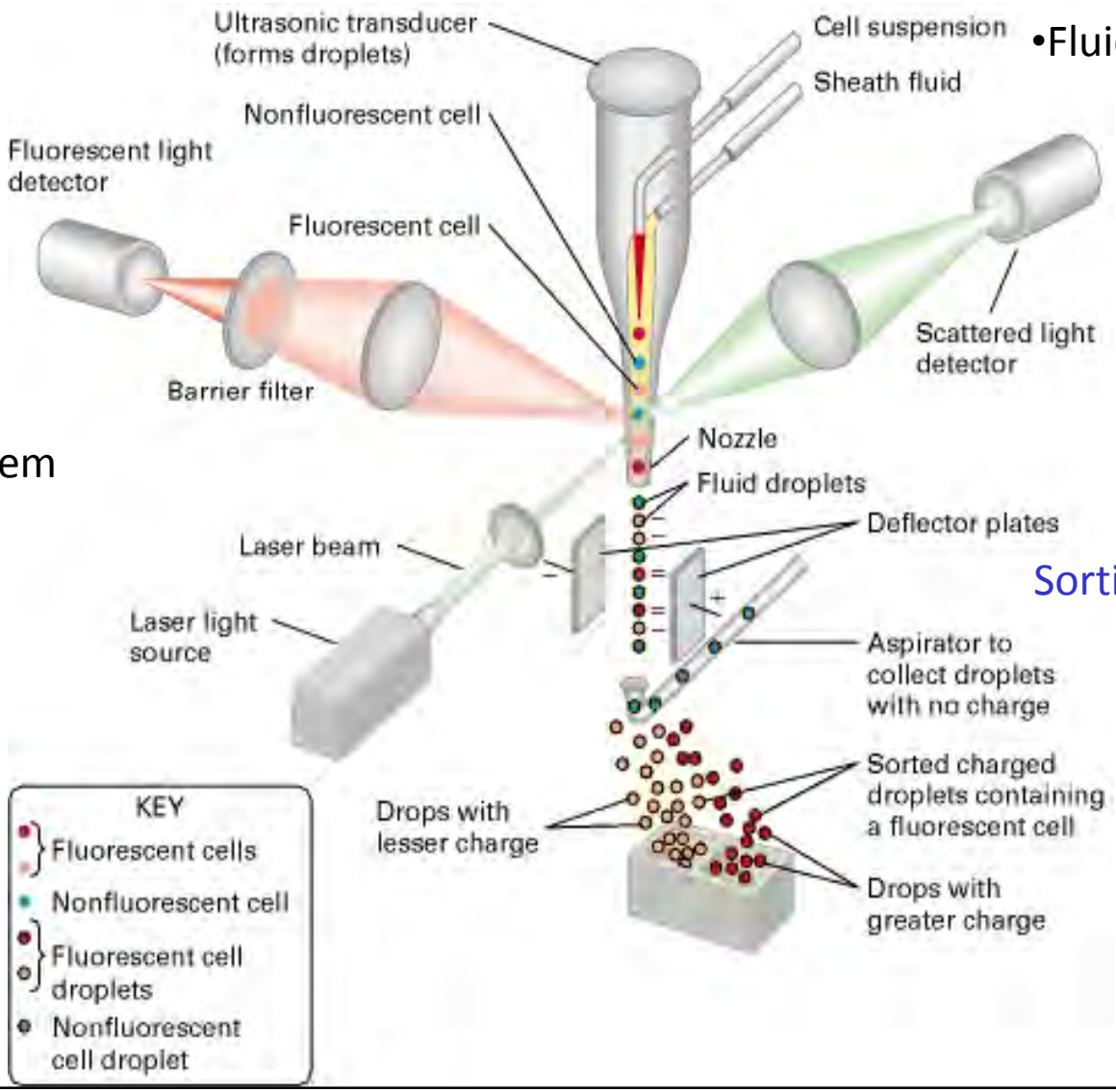
# *Flow Cytometry Is Made Of*

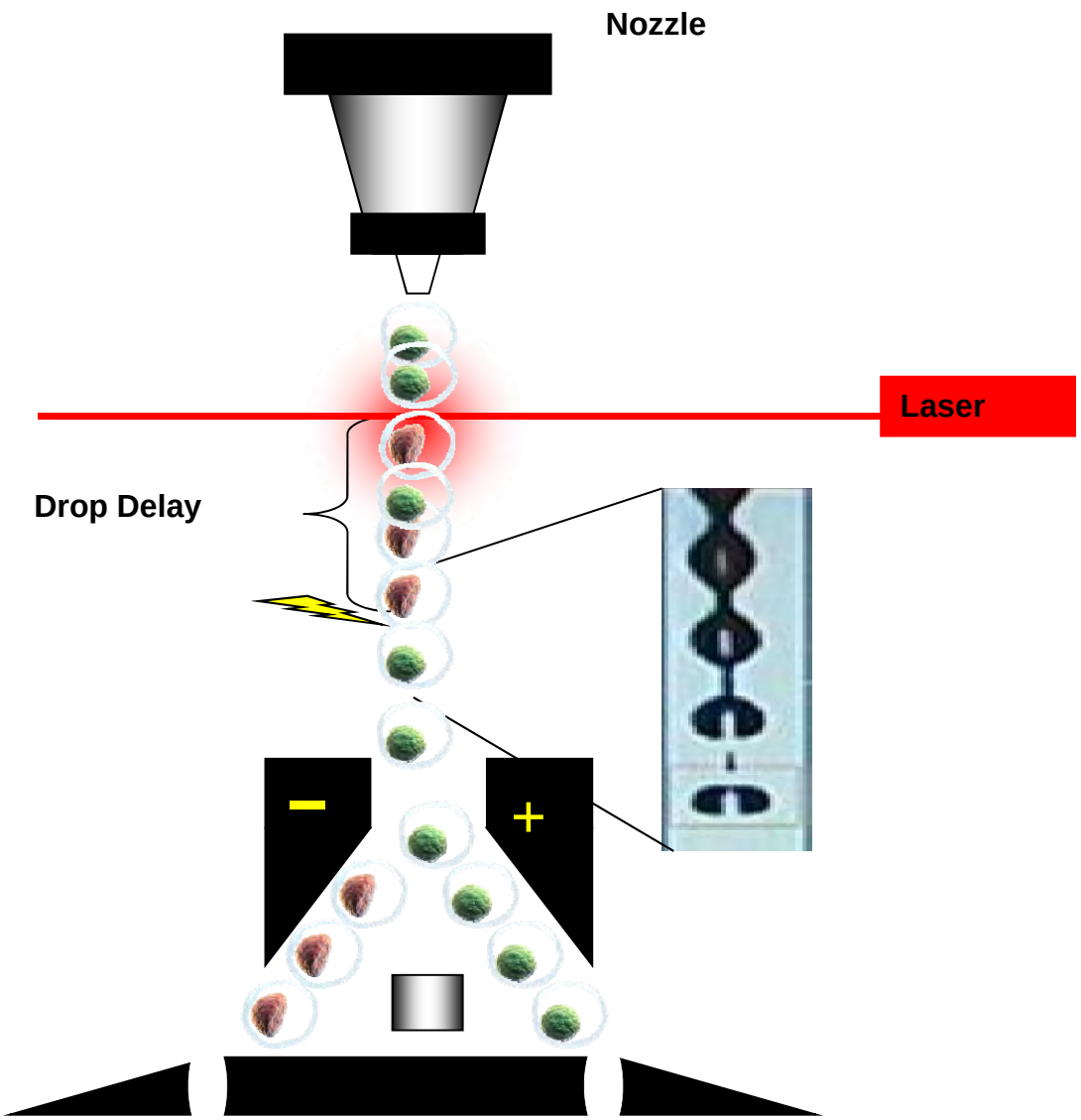
Electronic System

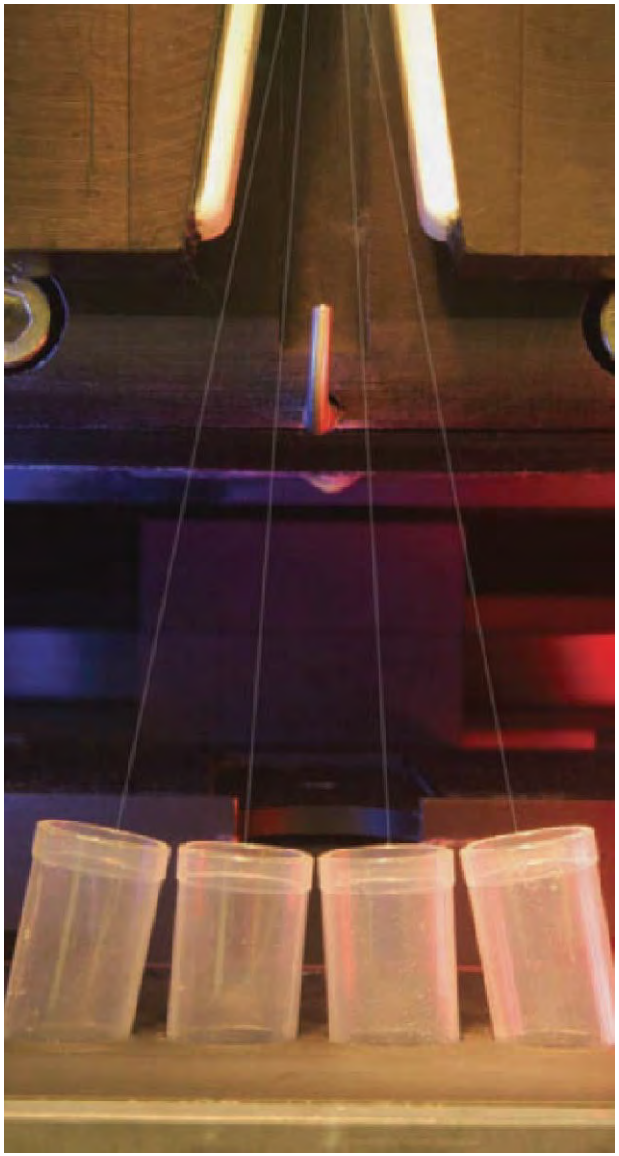
•Optical System

•Fluid System

Sorting System




Nozzle
Laser
Drop Delay
−
+



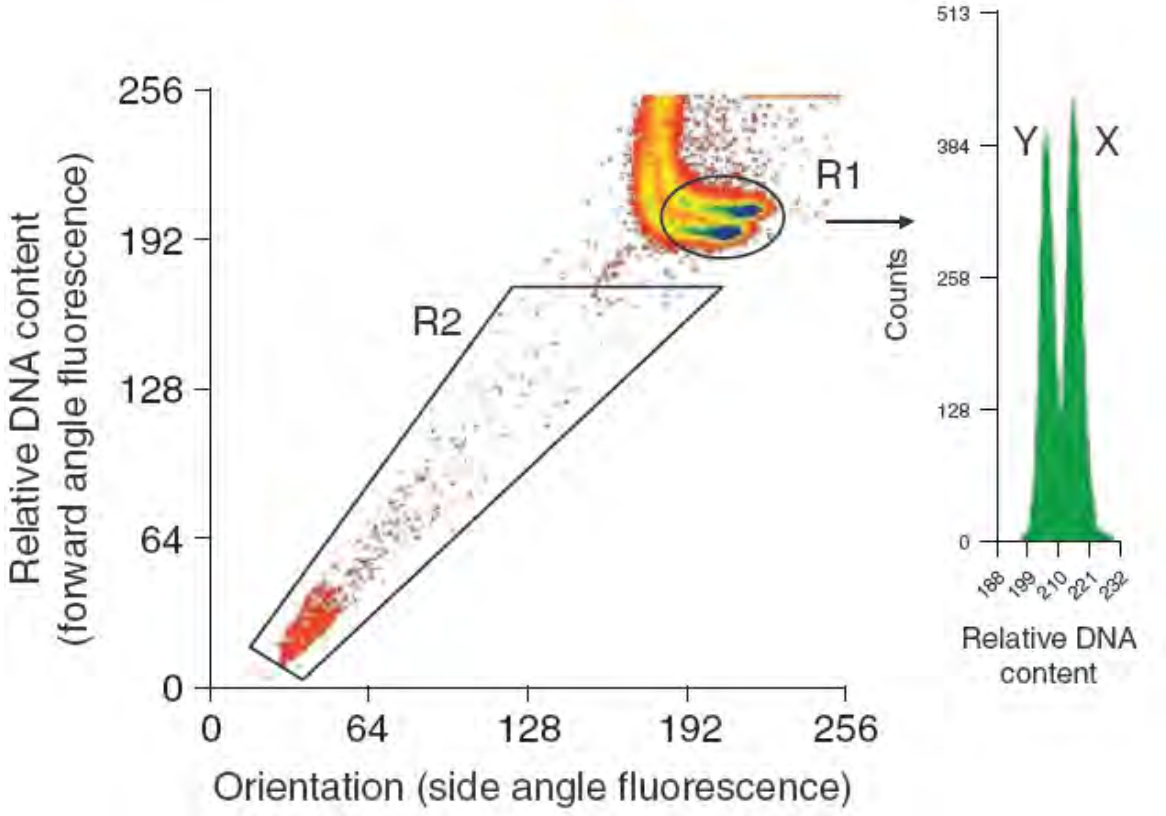

COHERENT
COHERENT

# Sperm sex selection

*Reproduction, Fertility and Development*, 2006, **18**, 319–329

## Development of sperm sexing and associated assisted reproductive technology for sex preselection of captive bottlenose dolphins

*J. K. O'Brien*[A,B,C] *and T. R. Robeck*[B]



CSIRO PUBLISHING

www.publish.csiro.au/journals/rfd

*Reproduction, Fertility and Development*, 2003, **15**, 367–375

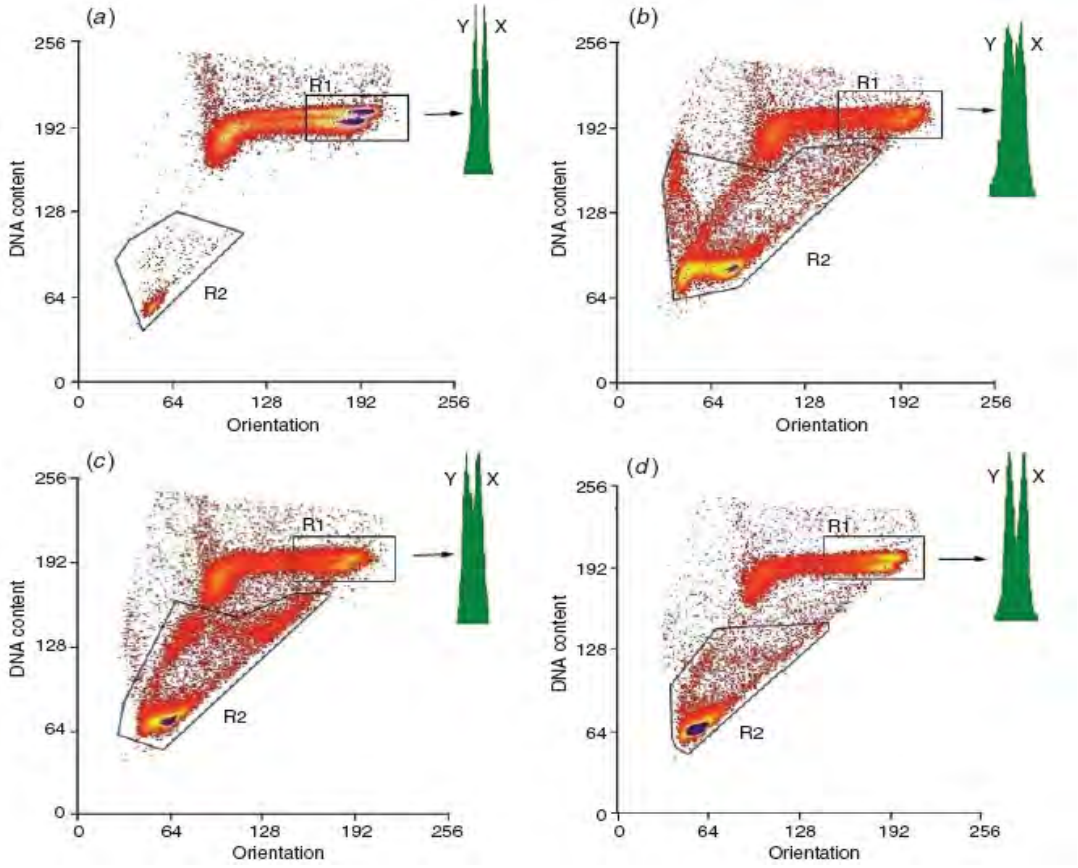
# Flow cytometric sorting of frozen–thawed spermatozoa in sheep and non-human primates

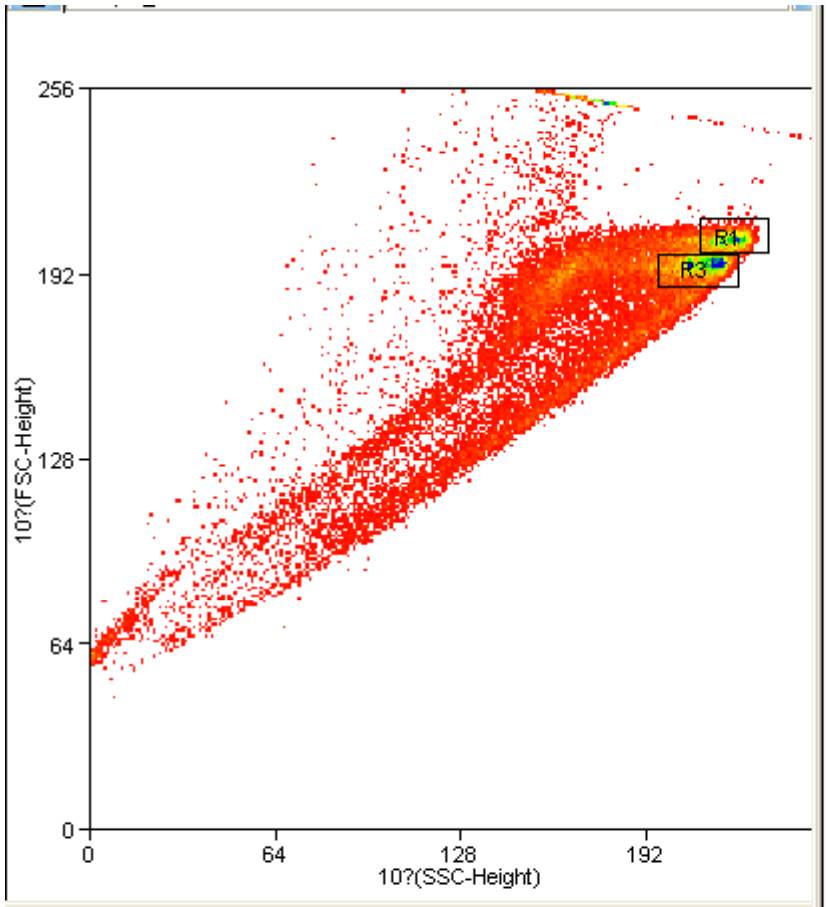
*J. K. O'Brien*[A,C], *F. K. Hollinshead*[A], *K. M. Evans*[B], *G. Evans*[A] *and W. M. C. Maxwell*[A]

[A]Centre for Advanced Technologies in Animal Genetics and Reproduction, Faculty of Veterinary Science, The University of Sydney, NSW 2006, Australia.

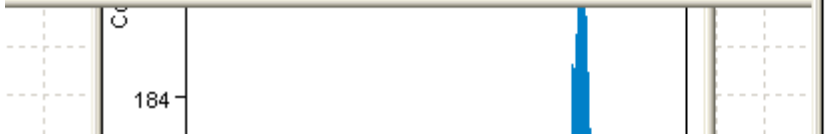
[B]XY Inc., Fort Collins, CO, USA 80523.

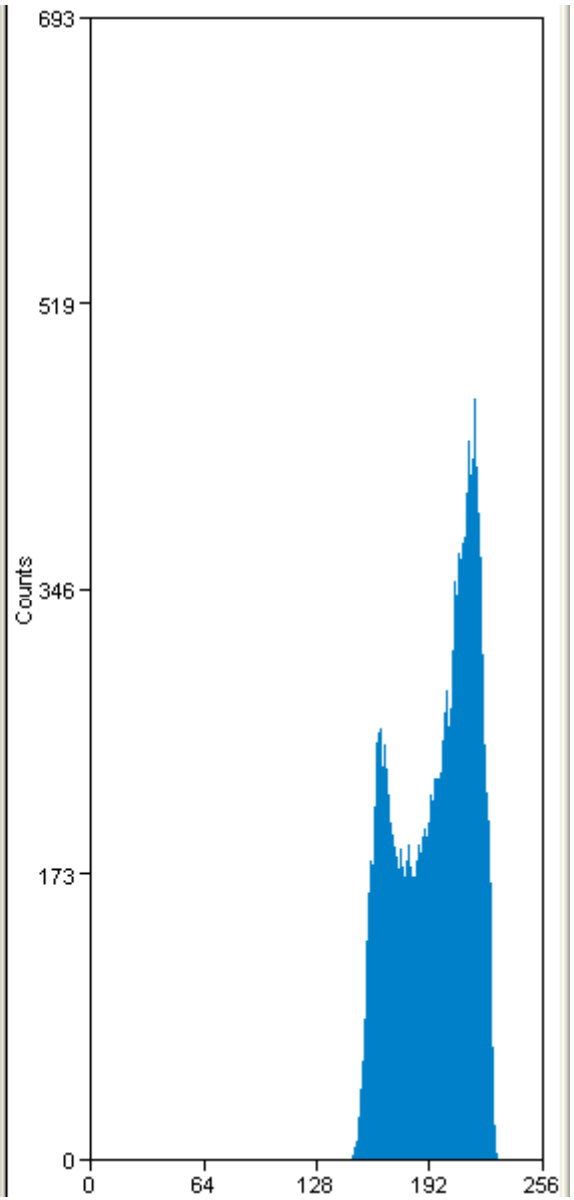
[C]To whom correspondence should be addressed. email: justineo@vetsci.usyd.edu.au






| Region | Count | % Hist | % All | Bounds | Mode |
|---|---|---|---|---|---|
| Total | 25369 | 100.00 | 100.00 | (0.00,255.00) (255.00... | |
| R1 | 2963 | 11.68 | 11.68 | (210.00,210.00) (234.... | |
| R3 | 4930 | 19.43 | 19.43 | (196.00,198.00) (224.... | |



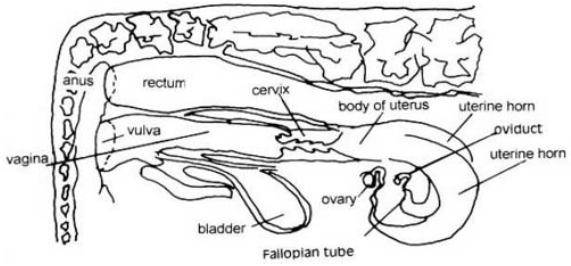
# AI with cryopreserved sexed sperm

Results of Trial 5—2001. Pregnancy rates in Holstein heifers following insemination of unsexed or sexed sperm

| Bull | Treatment | No. heifers | Pregnant (%) | Sexed as % of control |
|---|---|---|---|---|
| HO007 | $20 \times 10^6$ unsexed | 119 | (67%) | 78 |
| | $6.0 \times 10^6$ sexed | 75 | (57%) | |
| | $1.5 \times 10^6$ sexed | 101 | (48%) | |
| HO014 | $20 \times 10^6$ unsexed | 19 | (32%) | 72 |
| | $6.0 \times 10^6$ sexed | 59 | (24%) | |
| | $1.5 \times 10^6$ sexed | 24 | (21%) | |
| HO015 | $20 \times 10^6$ unsexed | 48 | (69%) | 57 |
| | $6.0 \times 10^6$ sexed | 58 | (40%) | |
| | $1.5 \times 10^6$ sexed | 92 | (39%) | |
| HO016 | $20 \times 10^6$ unsexed | 72 | (49%) | 77 |
| | $6.0 \times 10^6$ sexed | 61 | (34%) | |
| | $1.5 \times 10^6$ sexed | 81 | (40%) | |
| Average | $20 \times 10^6$ unsexed | 263 | (62%)[a] | 70 |
| | $6.0 \times 10^6$ sexed | 246 | (41%)[b] | |
| | $1.5 \times 10^6$ sexed | 288 | (43%)[b] | |

[a,b]Means without common superscript letters differ ($P < 0.05$).

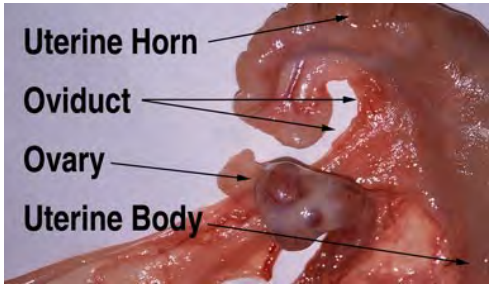
選性精液每劑AI精子數量對懷孕率的影響




Results of Trial 6—2000. Pregnancy rates in lactating Angus cows following insemination of unsexed sperm deposited into the uterine body or sexed sperm deposited into either the uterine horns or uterine body

| Treatment/site | No. cows | No. (%) pregnant day 60 | No. (%) calved | % Male |
|---|---|---|---|---|
| $20 \times 10^6$ unsexed/body | 21 | 16 (76%)[a] | 15 (71%) | 53 |
| $3.0 \times 10^6$ sexed/body | 42 | 24 (57%)[a,b] | 23 (55%) | 91 |
| $3.0 \times 10^6$ sexed/horn | 42 | 21 (50%)[b] | 21 (50%) | 90 |

[a,b]Means without common superscript letters differ ($P < 0.05$). There were no significant treatment differences in calving rates. However, calf sex for sexed treatments differed from the unsexed control ($P < 0.01$).




選性精液AI時置放位置對懷孕率的影響

*G.E. Seidel et al 2008*

# IVF of bovine embryos using sex-sorted sperm
# 利用選性精液進行牛胚體外生產

Means (± S.E.M.) for rates of embryo cleavage and blastocyst development from unsorted and sex-sorted sperm using ovaries obtained from anonymous donor cows at a commercial abattoir

| | Unsorted spermatozoa | Sex-sorted spermatozoa |
|---|---|---|
| Total no. of oocytes | 3312 | 1577 |
| No. of replicates | 24 | 19 |
| No. of oocytes per replicate | 138 | 83 |
| Cleavage rate (%) | 67.3 ± 3.5 | 65.0 ± 3.6 |
| Blastocyst development rate (%) | 20.1 ± 2.9 a | 12.2 ± 2.3 b |

Within a row, means with different letters differ ($P < 0.05$).

*R.D. Wilson et al 2006*

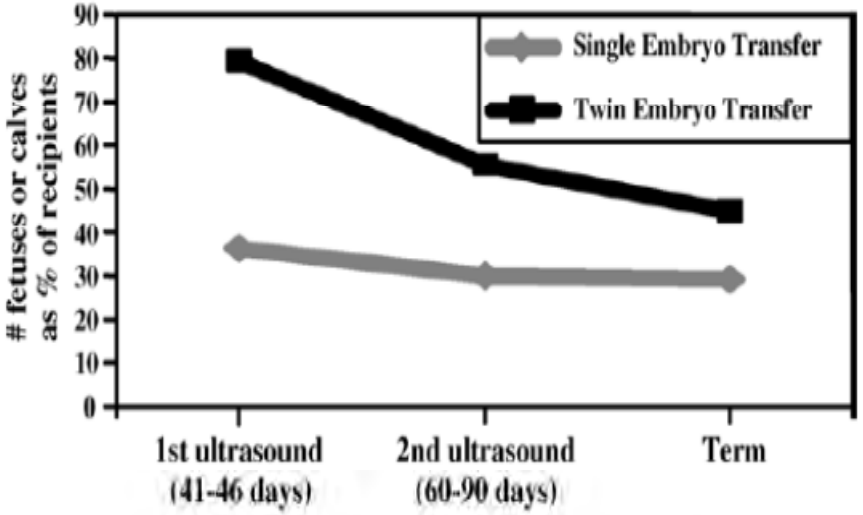



Fig. 1. Offspring production per recipient [29].

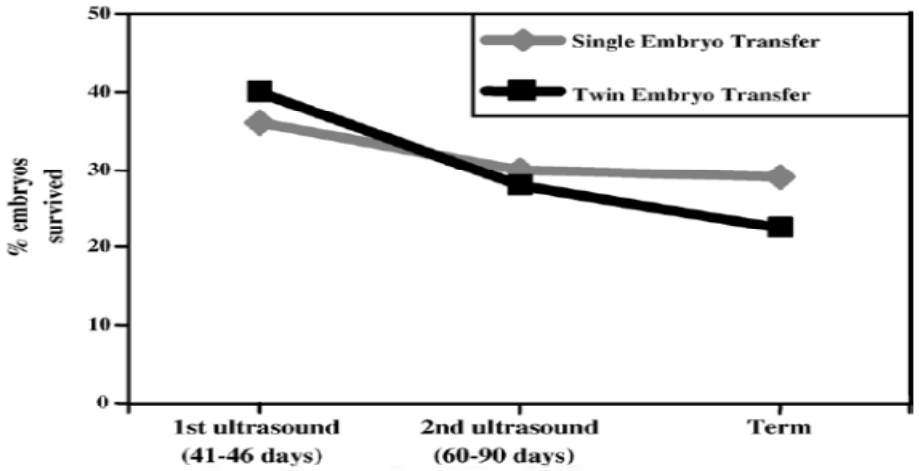



Fig. 2. Individual embryo survival [29].

*M.B. Wheeler et al. / Theriogenology 65 (2006) 219–227*

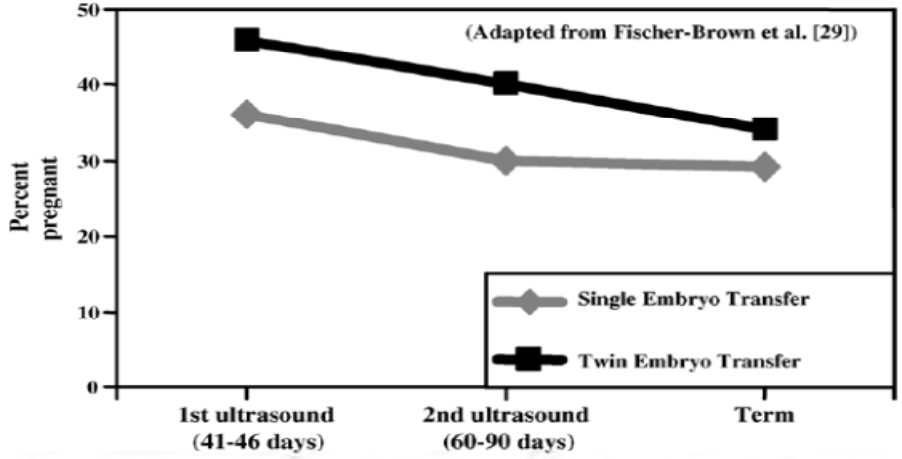



Fig. 3. Proportion of pregnant recipients irrespective of number of offspring [29].

性别控制胚可一次移置2個以增加代理孕母的懷孕率

*M.B. Wheeler et al 2006*

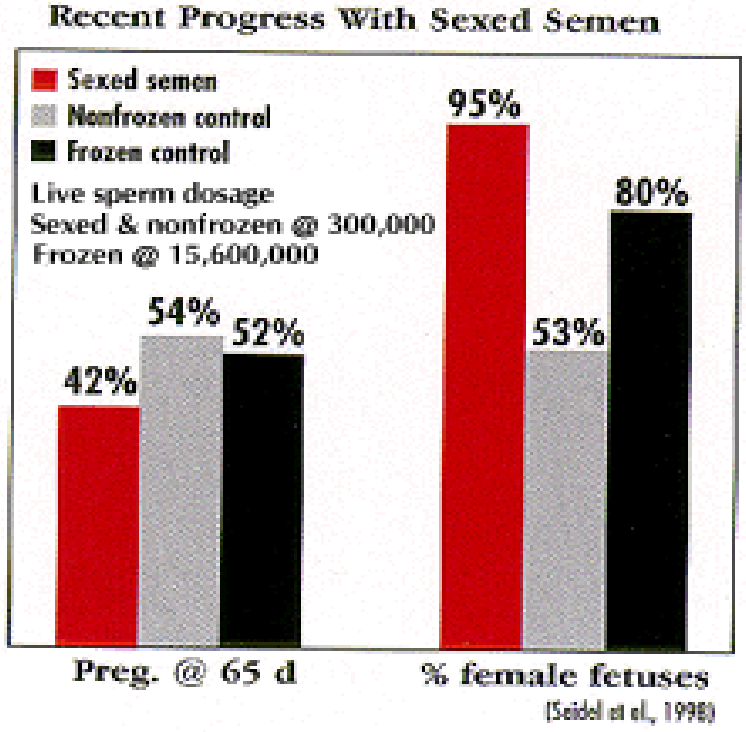
# One very appealing attribute of using flow-sorted sperm for IVP is that considerably fewer sperm are needed for IVF

- lower fertilization rates
- lower cleavage rates
- lower blastocyst rates
- lower pregnancy rates
- partial capacitation of the sperm
- dilute sperm samples
- sire variation

IVF相對使用較少量的精子就可受精 600隻/每個卵

# AI with cryopreserved sexed sperm

- lower fertility of sorted sperm
- lower survival of sorted sperm after cryopreservation
- reduced number of sperm that could be separated in a specified time period



# Conclusions

- Optimization the parameters (temperature, primers and cycles) for the PCR procedure made the present method rapid and reliable. Accuracy of sex prediction was 100%.

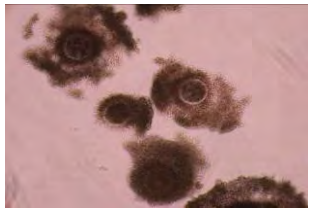
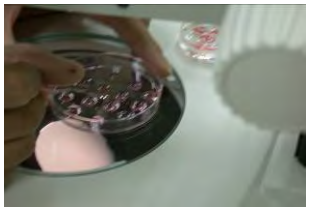
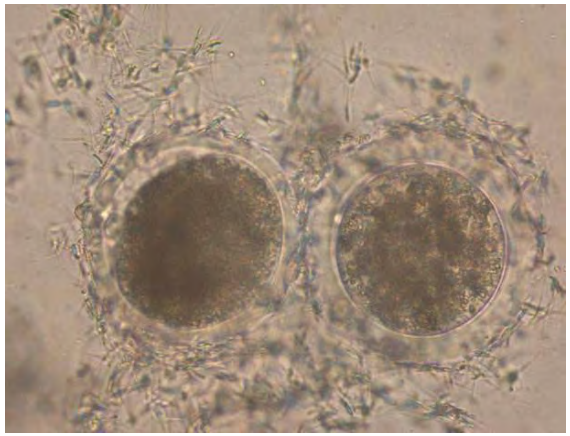
- Selective developmental arrest of male embryos induced by high-titer H-Y antisera. Under these conditions, selective embryonic developmental arrest may prove to be a commercially viable non-invasive method for sexing embryos.

- Sperm sorting by flow cytometer provides a powerful tool for artificial insemination and production of predefined sexed embryos but, an accurate verification of the yield of sperm separation remains essential for a field application of this technique or for improvement and validation of other related semen sexing technologies.

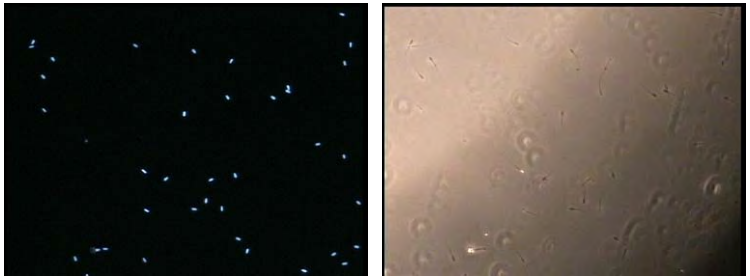
# IVF of bovine embryos using sex-sorted sperm

ovocyte collection and In vitro maturation ⇨ Few sperm fertilization ⇨ Embryo transfer

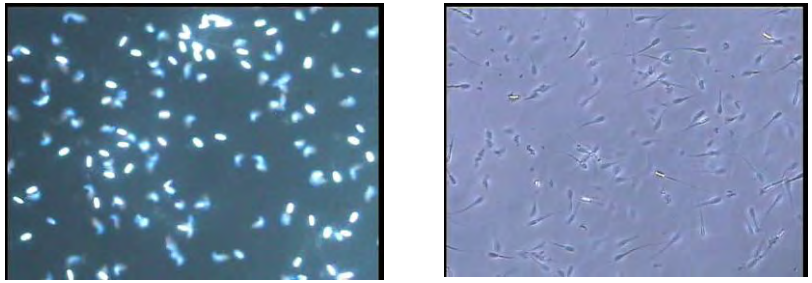


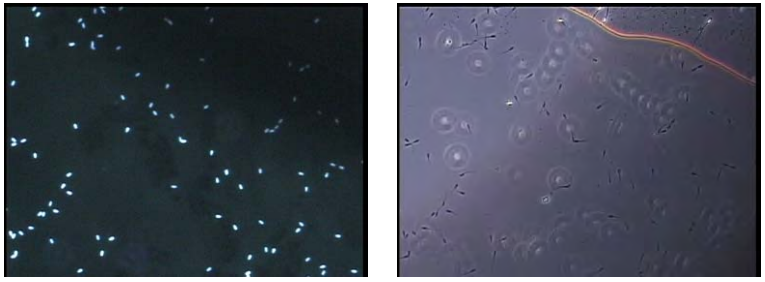

# Sperm standing before sorting



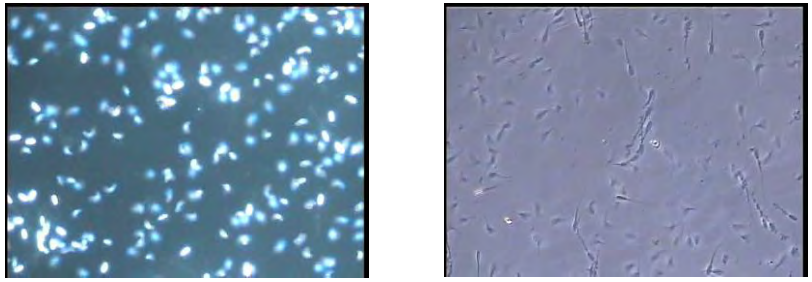
染色後 6 hours小時



染色後0 hours小時



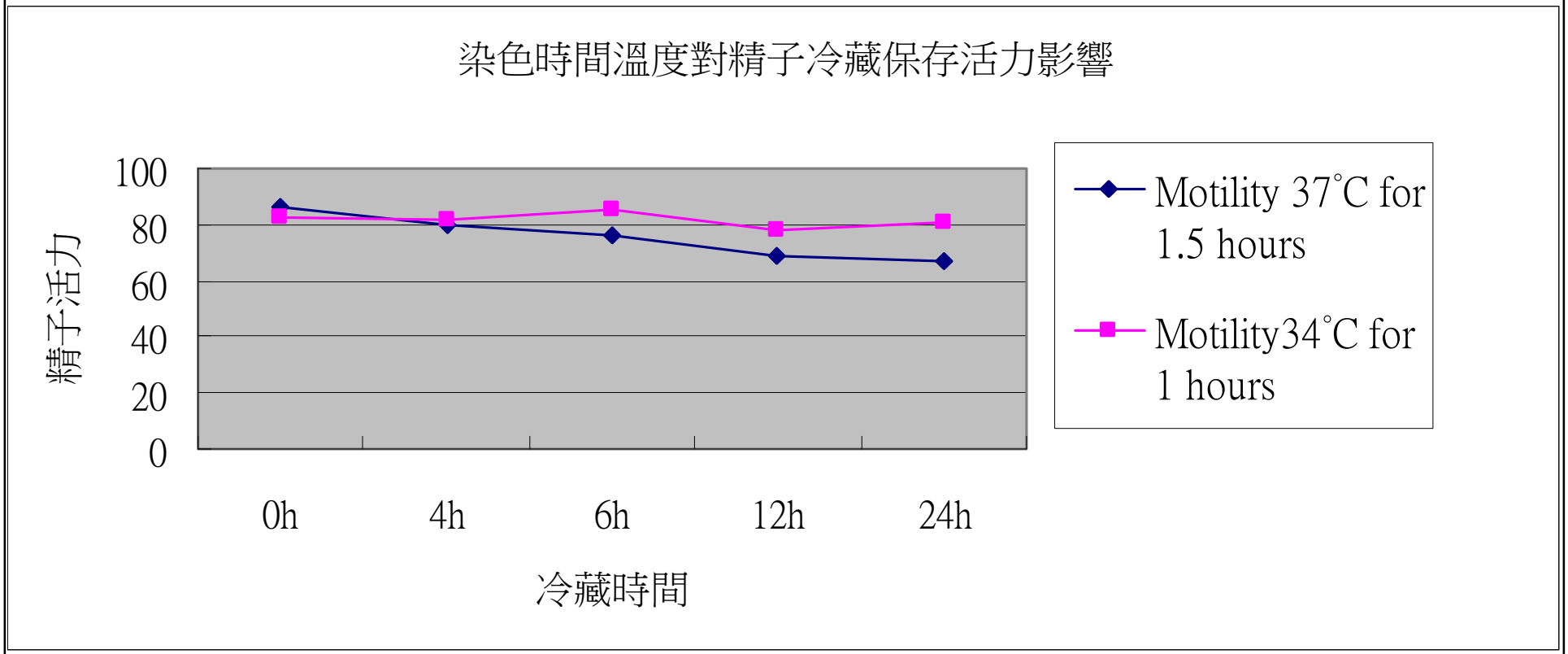
染色後12 hours小時



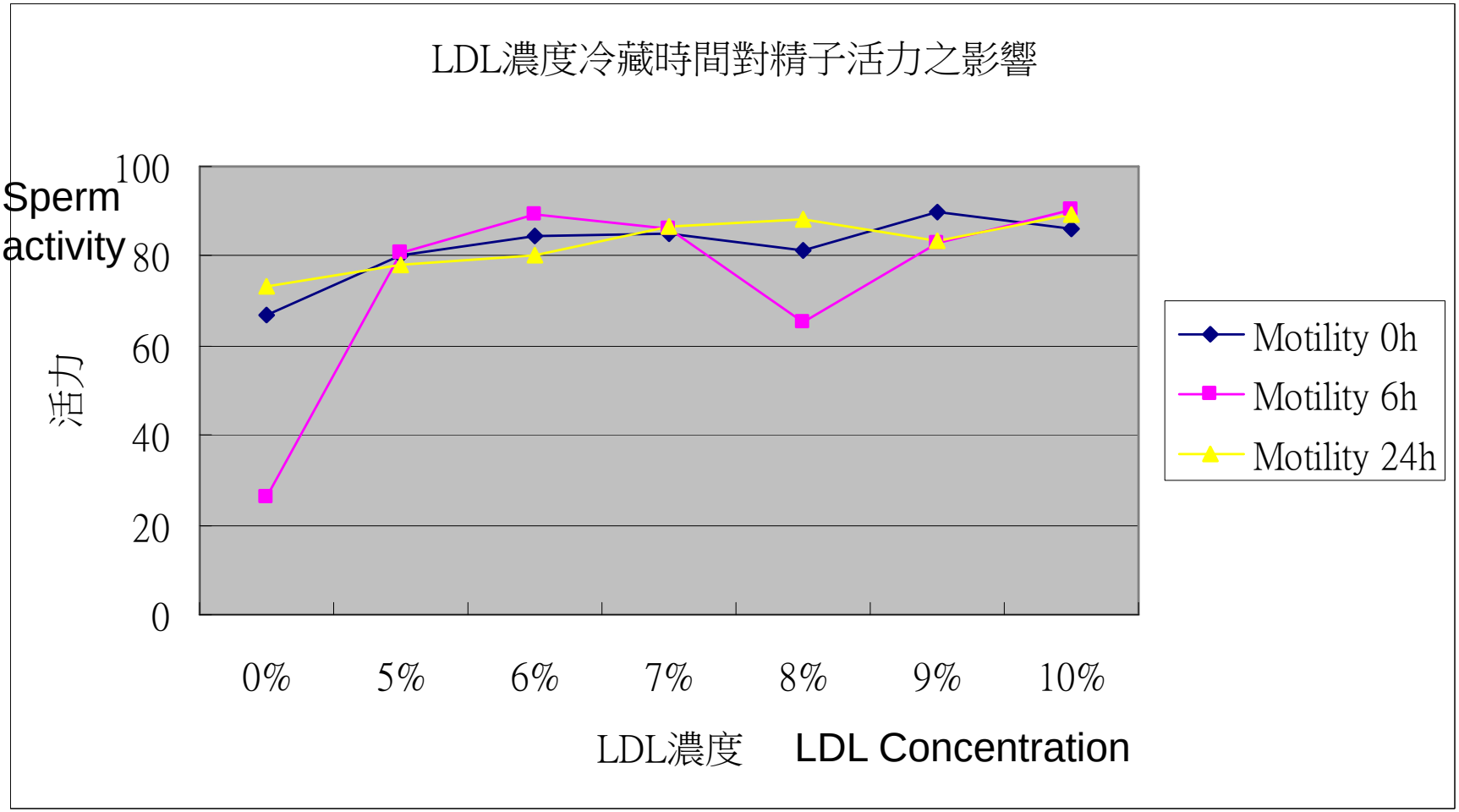
染色後24 hours小時 Fluorescence Continues obviously

8.12 mM Hoechst 33342 solution and incubated at 34° C for 1 hours

# Sperm standing before sorting



Before the sperm sex separation, 5% LDL Has the protection function



# Application of sexed sperm

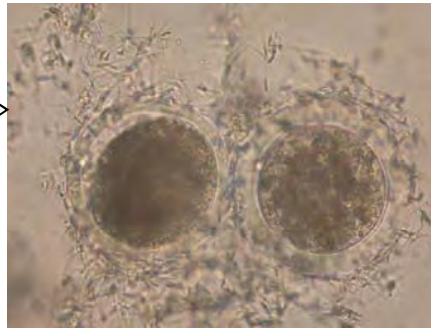
Artificial insemination

選性精子人工授精

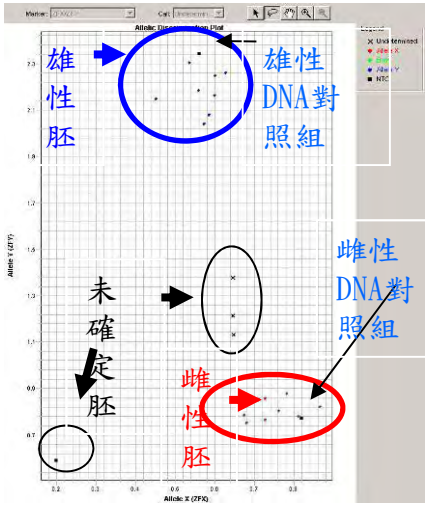
理想性別仔畜生產

sexed sperm

In vitro embryo production

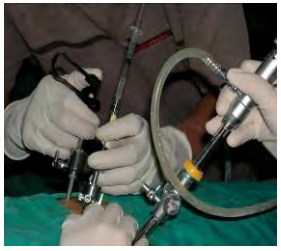


IVF

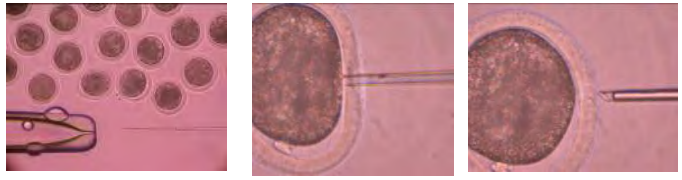



RT PCR 胚性別鑑定

Few sperm fertilization



Endoscope insemination



Intracytoplasmic sperm injection,ICSI

代理孕母胚移置


Thanks for Your Attention