水鹿人工授精班
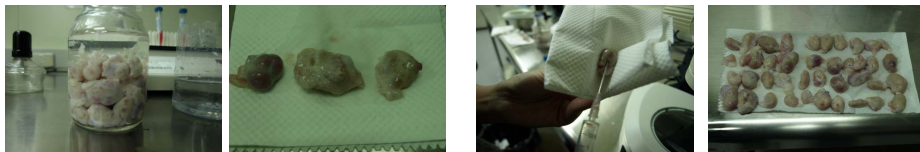
家畜選性繁殖技術

2015 9月 生理組曲鳳翔

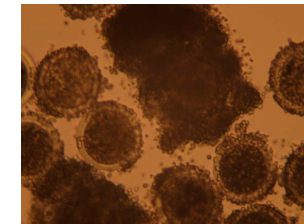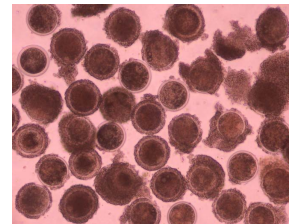## Subject

- Sex preselection of bovine reproduction

Sexing preimplantation embryos by PCR

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

Overview of sexing sperm

AI with cryopreserved sexed sperm

IVF of bovine embryos using sex-sorted sperm

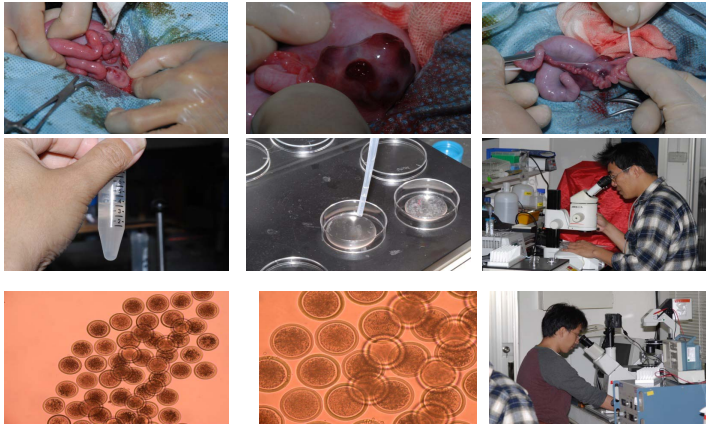---

Collection and in vitro maturation of goat oocytes

In vitro maturation of goat oocytes
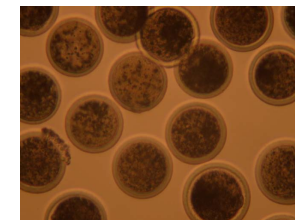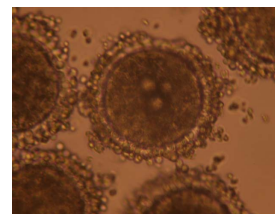
Collection in vivo maturation goat oocytes

Collection and in vitro maturation of bovine oocytes
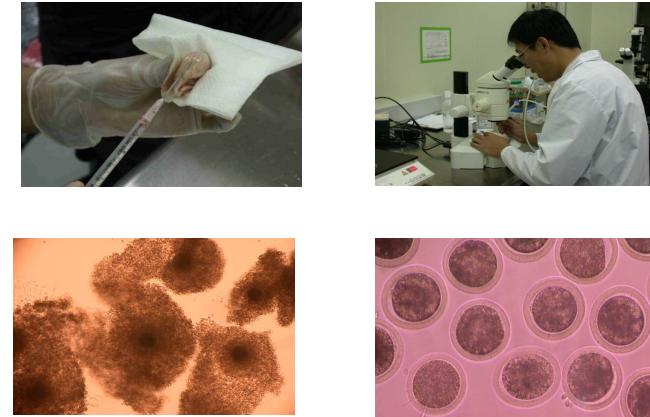
Sperm microinjection

IntraCytoplasmic Sperm Injection，ICSI
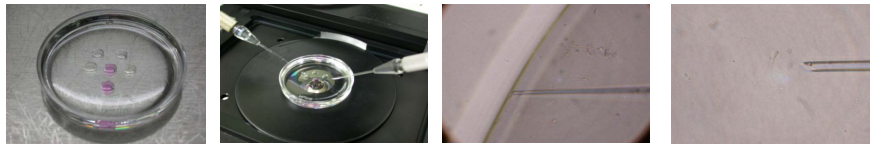
ICSI manipulation drop
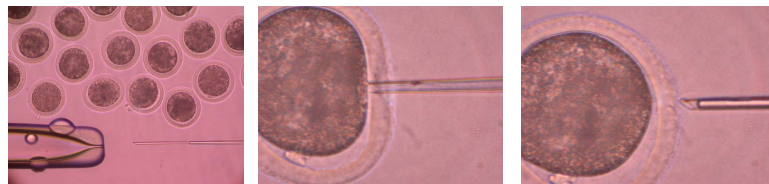
maturation oocyte ICSI

Sexing sperm in vitro fertilization

- Using sexing sperm to carry on the cow extra-embryonic fertilization experiment altogether to carry on 18 times, produces system sex control embryo 693, splits 2 cell above 223, grows to pouch embryo 39 (17.4%), normal sperm cell external fertilization growth to pouch embryo proportion (21.9%) slightly low, the transpose 21 individualities do not control the embrionic transpose to be pregnant the mother in 13 agents.

Sexing sperm microscopic exam and counting
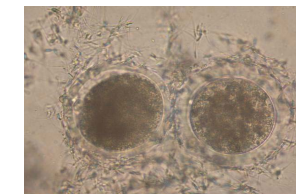
Sexing sperm in vitro fertilization

The goat endoscope few sperms AI experiment

- (1/6th artificial insemination normal zoosperm quantity) carries on by the few sperms by the endoscope way pours into oviduct of the palace tube joint spot, carries on 14 first time, is pregnant 10 (being pregnant rate 71.4%), is divided exempts 20 lambs, average each bears young two heads.

Pursues the fertilization spot

peritoneoscope few sperm fertilizations

The ewe gives birth to four lamb

羊號 tnumber	發情時間 Estrus time	孕診結果 pregnant	產仔數 lamb
827	11:05	+	2♀2♂
842	11:30	+	1♀1♂
744	11:30	+	2♂
851	16:30	+	1♀1♂
686	16:30	+	1♀1♂
843	8:30		
853	8:44		
548	11:00	+	1♀1♂
H1895	11:01		
638	11:00	+	1♀1♂
792	11:00	+	1♂
517	11:00	+	1♂
780	11:01		
828	11:02	+	1♂

Embryo in vitro culture of goat

GOEC Coculture to SOF influence of the system Parthenogenetic Activation goat embryo growth ability

Culture system	No. of embryo	No. of activated oocytes developed to (%)			
		2-cell	8-cell	16-cell	B
Sof+GOEC	97	65(67.0%)	28(43.1)	22(33.8)	21(32.3)
Sof	82	52(63.4%)	21(40.4)	18(34.6)	16(30.8)
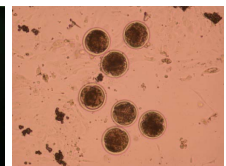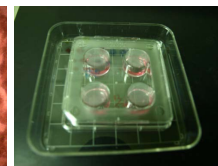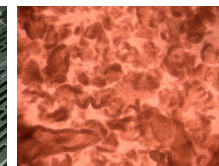
Bovine Embryo in vitro culture
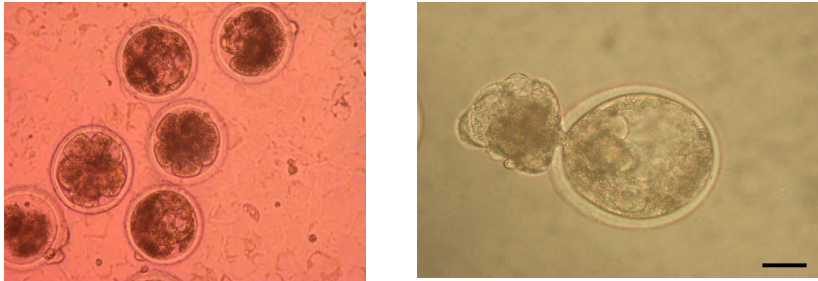
Cumulous cell coture with bovine embryo

Vitrification Cryopreservation in Embryos
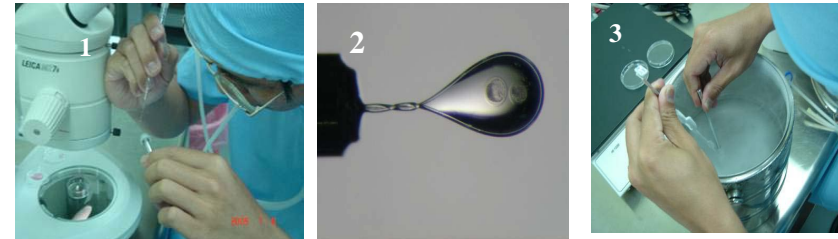
VS-A (Vitrification solution A) group: 10%EG+10%DMSO. 45 seconds

VS-B (Vitrification solution B) group: 15%EG+15%DMSO+ 0.5M sucrose. 30 seconds

1038- freezing embryo defrosting transpose condition

1038- freezing embrionic growth condition

after 1038- defrosting, embryo's restoration condition
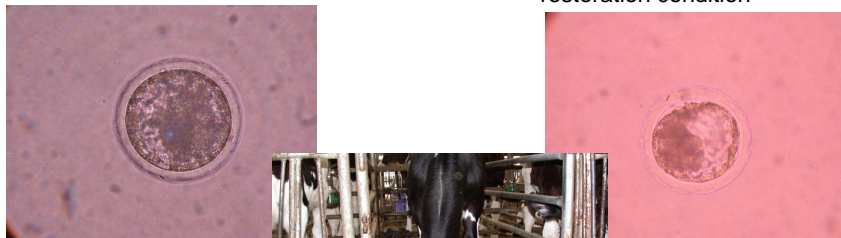
after 1038 in vivo embryo freezing defrosts, transposes young cow of the childbirth (1483)

Application of sexed sperm

2011 11/ 21 Feng Hsiang chu

Physiological department

Outline

■ 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

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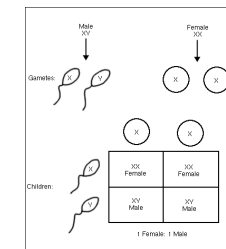
Sexing preimplantation embryos by PCR
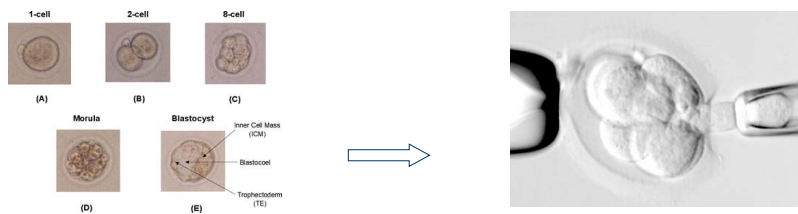
Embryos in vitro
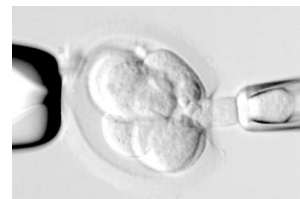
micromanipulation

PCR mechanism
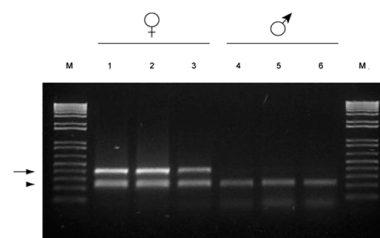
Gel electrophoresis

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.

Slide 1 (top-left)

237bp

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 1000bp 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

Slide 2 (top-right)

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.

圖四、以肉眼檢視LAMP反應結果。(A) 可見光下直接觀察。(B) 加入EtBr染劑在可見光下觀察。(C) 加入EtBr染劑在紫外光下觀察。

Slide 3 (bottom-left)

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'-ATTGCATGTGGGAAGAACTGTAG-3'

Male-female common primers
Inner primer F   5'-GAGGAACATTGGCTTCTGGACAAGCTGGGGATTGCTCT-3'
Inner primer B   5'-AGTGGAAGCAAAGAACCCCACCCAGTGAGCTCCAA-3'
Outer primer F   5'-CATGGCCTAGAGACCAATC-3'
Outer primer B   5'-CATGGCCTAGAGACCAATC-3'
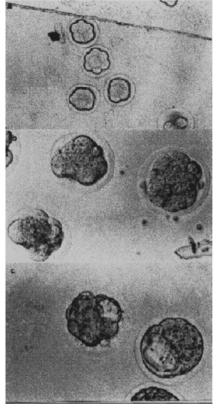Loop primer F   5'-CCTAGATGAGGTCTATTGGC-3'
Loop primer B   5'-CTGCTCTCGAATTGTGACG-3'

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

Slide 4 (bottom-right)

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

Slide 1

M.F.P.D.-T. Ramalho et al./Theriogenology 62 (2004) 1569–1576   1571

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.

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).

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

Slide 2

Overview of sexing sperm

Slide 3

8,000-6,000 B.C.

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

1780

First well-documented artificial insemination using dogs.

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.

Slide 4

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
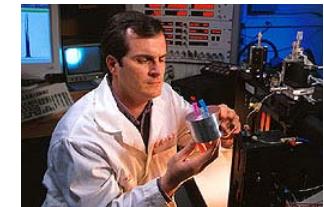
D.L. Garner / Theriogenology 65 (2006) 943–957
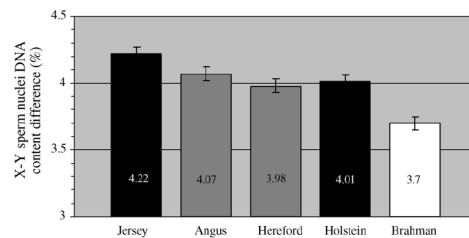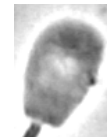
不同品種牛隻其X-Y精子染色體量的差異也不同
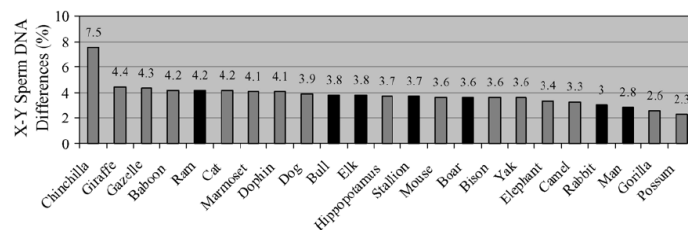
D.L. Grarner 2006

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) Head sagittal section	9.1	9.0	8.1	7.7	7.7	7.0	6.5	4.6
Width (μm) Head profile	4.7	5.0	4.0	4.5	3.2	3.5	3.4	3.2
Area (μm²)	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 (μm²) × X-Y Sperm DNA difference (%).

高雄場與生理組 2010

D.L. Grarner 2006

Slide 1 (top-left)

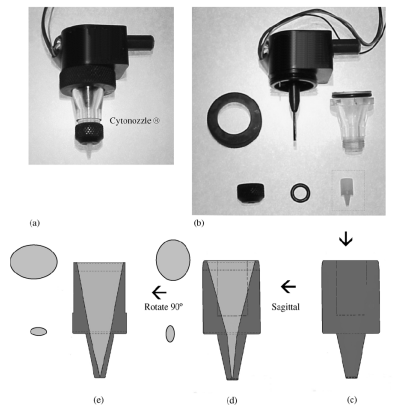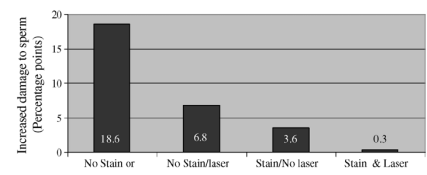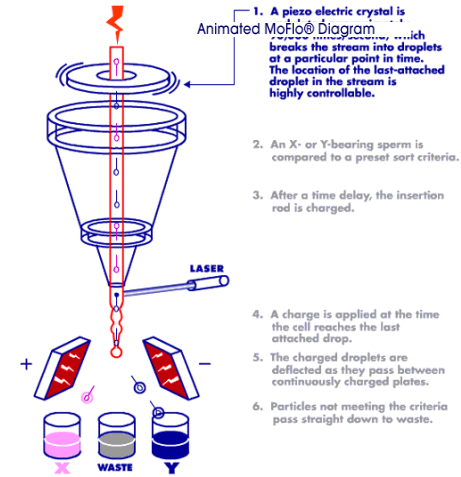D.L. Garner /Theriogenology 65 (2006) 943–957 949

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

Slide 2 (top-right)

Animated MoFlo® Diagram

1. A piezo electric crystal is ... which breaks the stream into droplets at a particular point in time. The location of the last-attached droplet in the stream is highly controllable.

2. An X- or Y-bearing sperm is compared to a preset sort criteria.

3. After a time delay, the insertion rod is charged.

4. A charge is applied at the time the cell reaches the last attached drop.

5. The charged droplets are deflected as they pass between continuously charged plates.

6. Particles not meeting the criteria pass straight down to waste.

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

## Slide 3 (bottom-left)

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
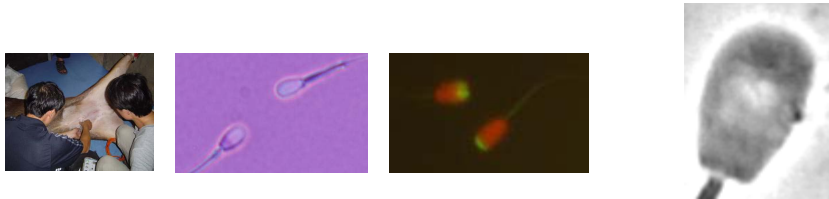- Purity mode
- Single mode

➢ CyClone deposition system

BD com **influx**

Slide 4 (bottom-right)

■ 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%
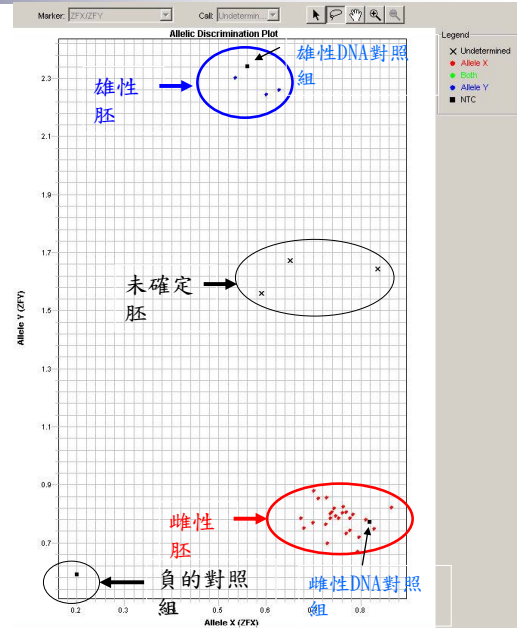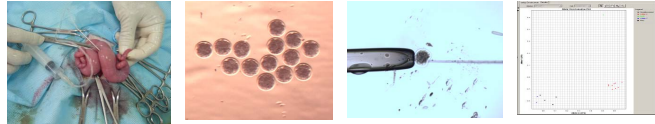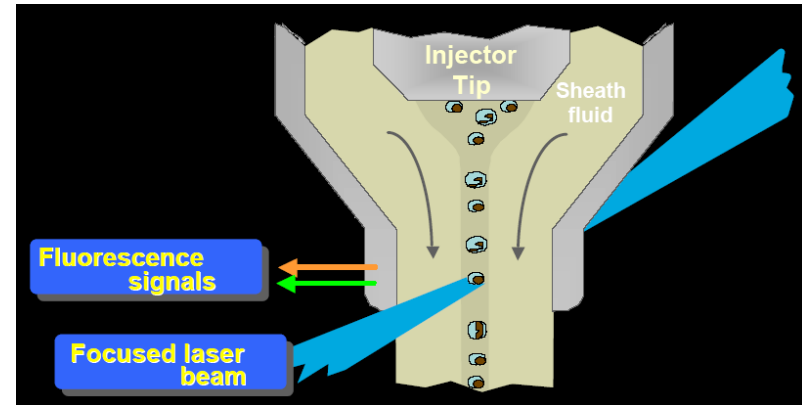
■ 水鹿精子型態及分離條件之評估冷藏保存技術建立（高雄場）

讓細胞在管路中排路隊
Hydrodynamic Focusing（流體動力聚焦）

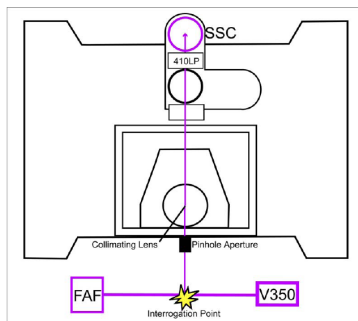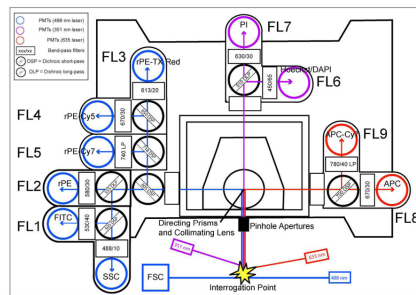
Sperm sorting V.S. cell Sorter差異
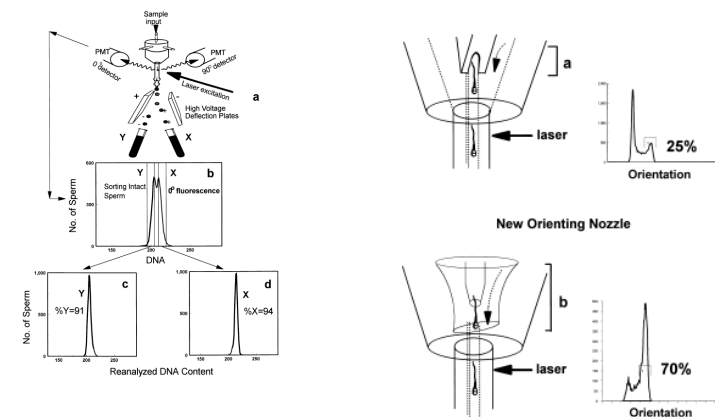
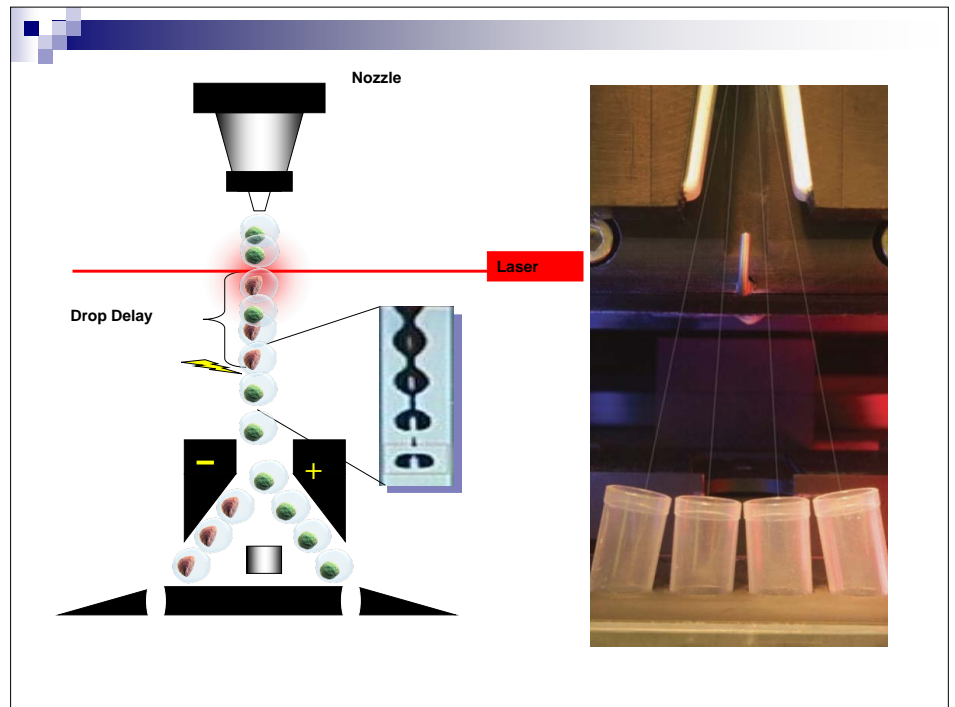1. Optical System diferent
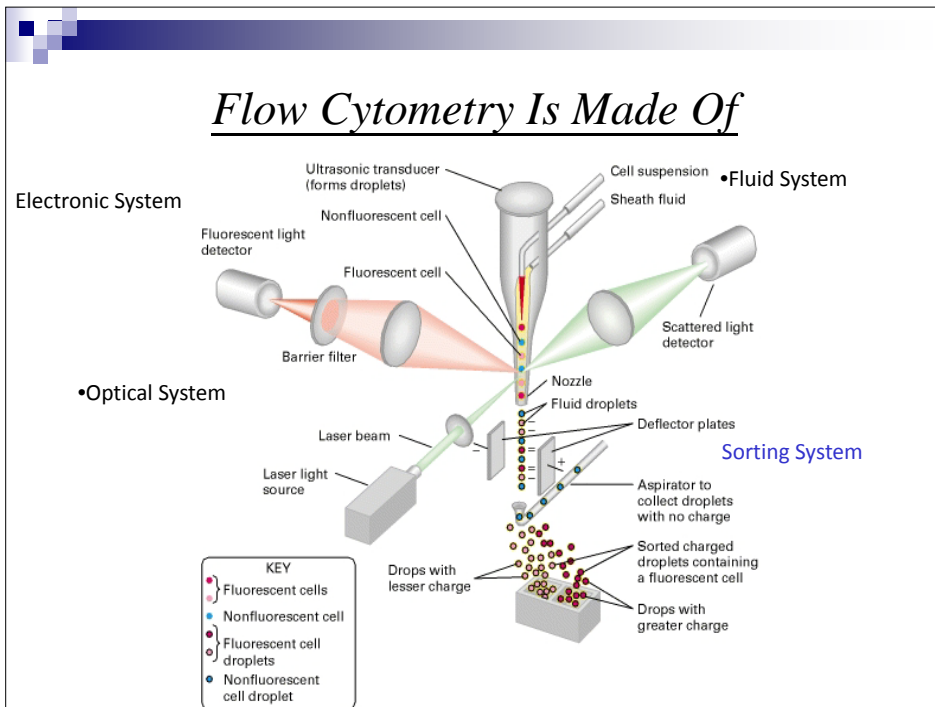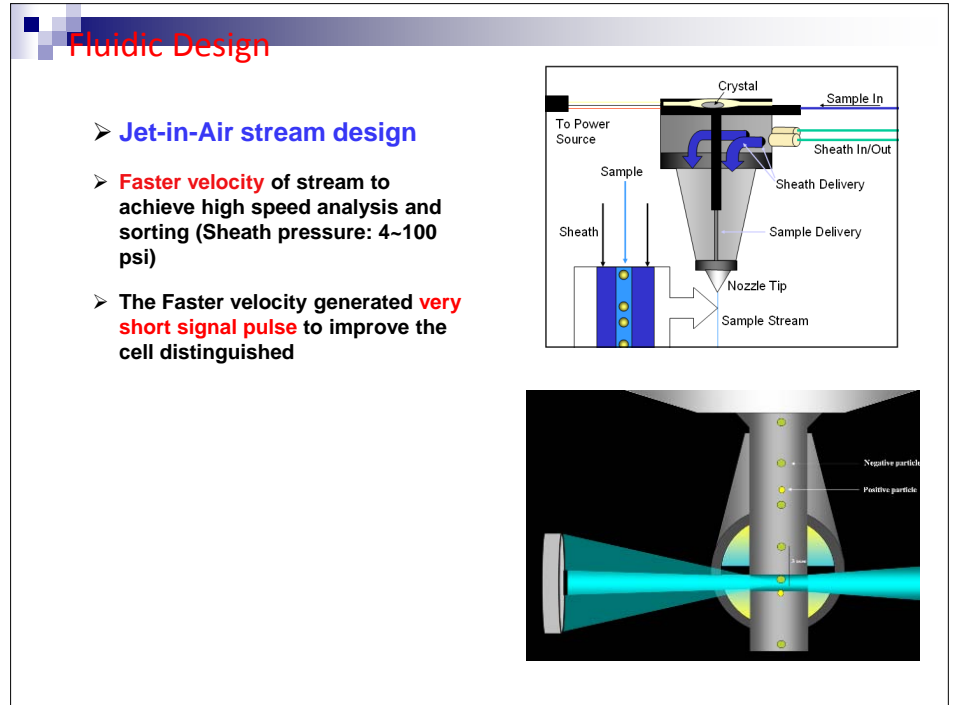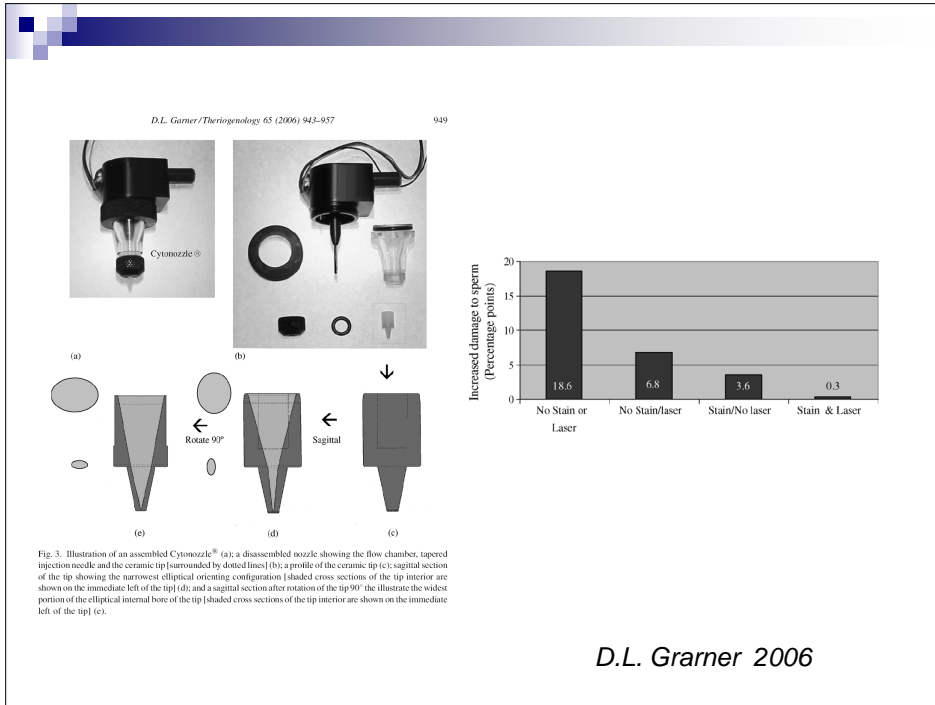
Sperm sorting

Sorter

Wave length 350

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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.

D.L. Garner / Theriogenology 65 (2006) 943–957   949

Cytonozzle®

(a)   (b)

Rotate 90°   Sagittal

(e)   (d)   (c)

Increased damage to sperm (Percentage points)

No Stain or Laser: 18.6
No Stain/laser: 6.8
Stain/No laser: 3.6
Stain & Laser: 0.3

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

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Flow Cytometry Is Made Of

Electronic System

•Fluid System

•Optical System

Sorting System

Sperm sex selection

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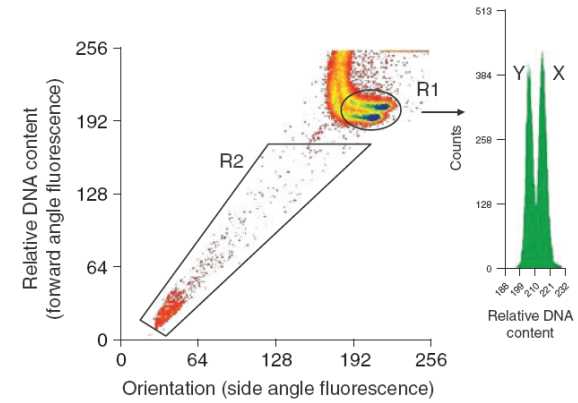
Flow cytometric sorting of frozen–thawed spermatozoa in sheep and non-human primates

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
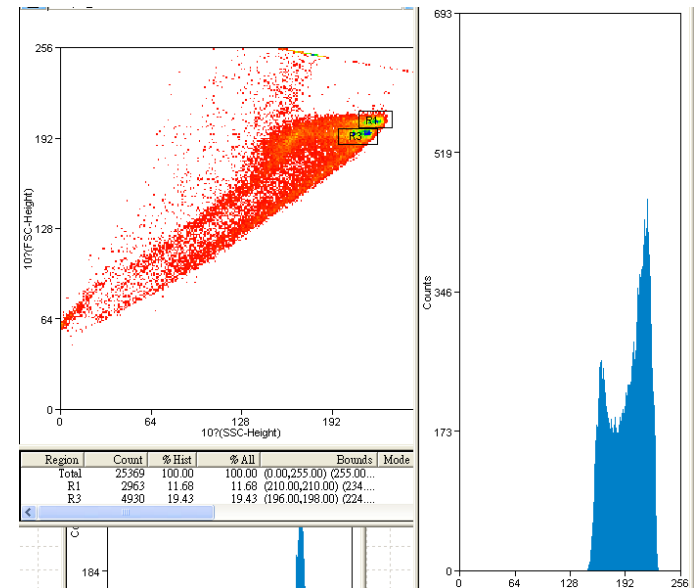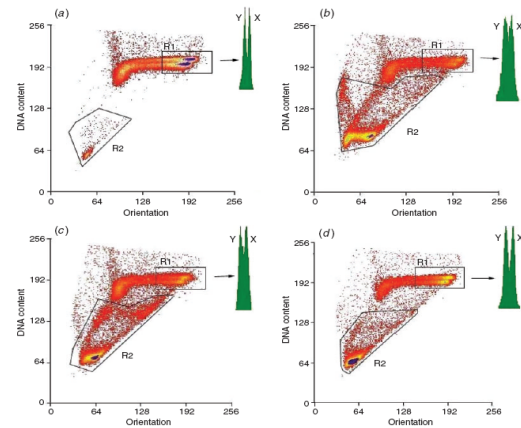
AI with cryopreserved sexed sperm

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	20×10^6 unsexed	119	(67%)	78
	6.0×10^6 sexed	75	(57%)	
	1.5×10^6 sexed	101	(48%)	
HO014	20×10^6 unsexed	19	(32%)	72
	6.0×10^6 sexed	59	(24%)	
	1.5×10^6 sexed	24	(21%)	
HO015	20×10^6 unsexed	48	(69%)	57
	6.0×10^6 sexed	58	(40%)	
	1.5×10^6 sexed	92	(39%)	
HO016	20×10^6 unsexed	72	(49%)	77
	6.0×10^6 sexed	61	(34%)	
	1.5×10^6 sexed	81	(40%)	
Average	20×10^6 unsexed	263	(62%)[a]	70
	6.0×10^6 sexed	246	(41%)[b]	
	1.5×10^6 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
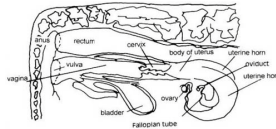
Results of Trial 6—2000. Pregnancy rates in lactating Angus cows following insemination of unsexed sperm deposited into the uterine body or sexed sperm deposited into either the uterine horns or uterine body

Treatment/site	No. cows	No. (%) pregnant day 60	No. (%) calved	% Male
20×10^6 unsexed/body	21	16 (76%)[a]	15 (71%)	53
3.0×10^6 sexed/body	42	24 (57%)[a,b]	23 (55%)	91
3.0×10^6 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
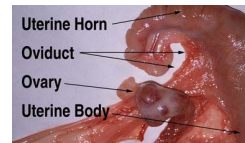
G.E. Seidel et al 2008

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

Means ($\pm$ 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 ($P < 0.05$).
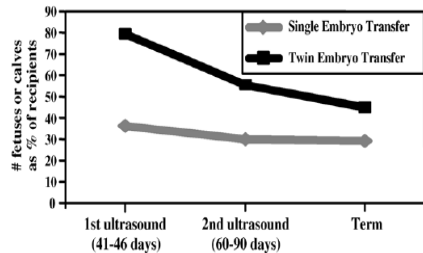
R.D. Wilson et al 2006

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

M.B. Wheeler et al./Theriogenology 65 (2006) 219–227
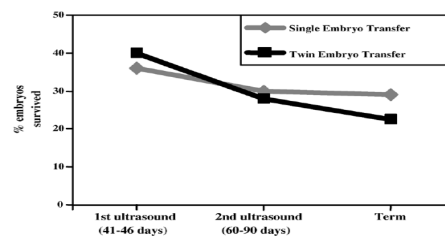
Fig. 2. Individual embryo survival [29].

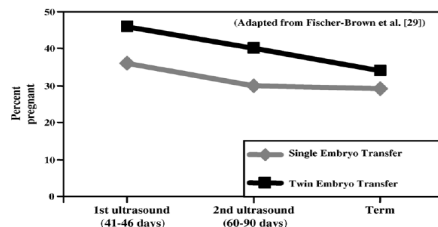(Adapted from Fischer-Brown et al. [29])

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

性別控制胚可一次移置2個以增加代理孕母的懷孕率

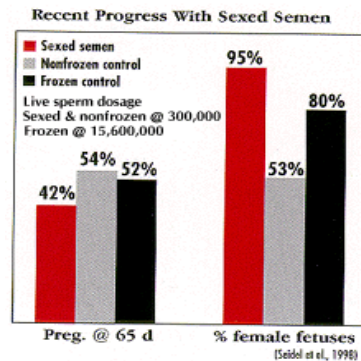*M.B. Wheeler et al 2006*

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
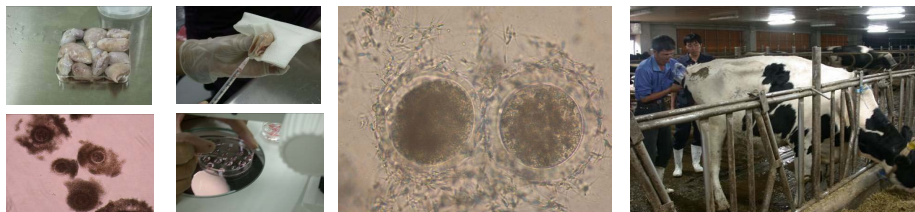

Recent Progress With Sexed Semen

- Sexed semen
- Nonfrozen control
- Frozen control

Live sperm dosage
Sexed & nonfrozen @ 300,000
Frozen @ 15,600,000

95%
54% 52%
42%
53%
80%

Preg. @ 65 d   % female fetuses
(Seidel et al, 1998)

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
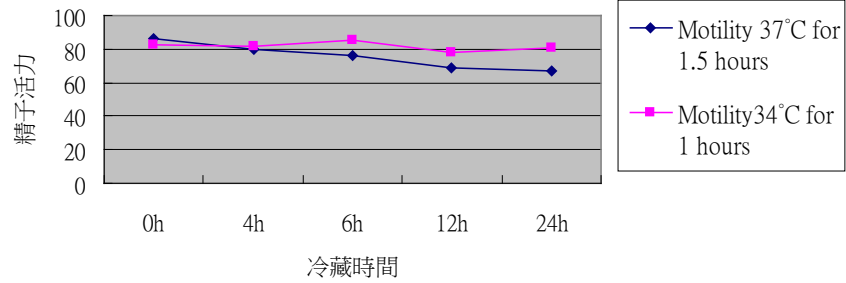and In vitro maturation ⟹ Few sperm fertilization ⟹ Embryo transfer

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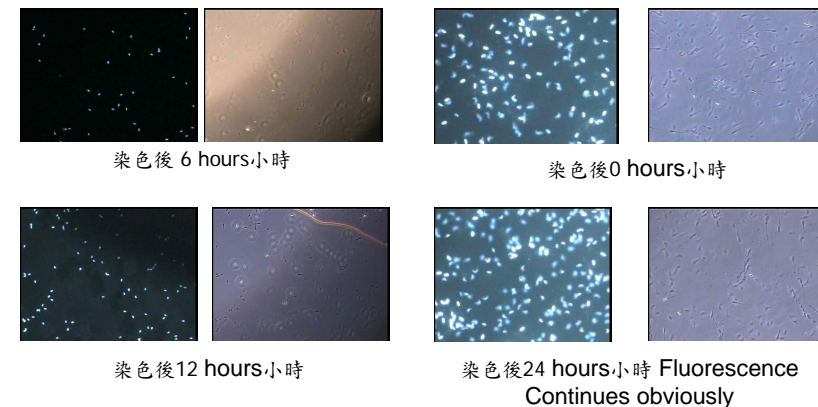
8.12 mM Hoechst 33342 solution and incubated at 34°C for 1 hours

Sperm standing before sorting

染色時間溫度對精子冷藏保存活力影響

Legend:
- Motility 37°C for 1.5 hours
- Motility 34°C for 1 hours

Y-axis: 精子活力 (0, 20, 40, 60, 80, 100)
X-axis: 冷藏時間 (0h, 4h, 6h, 12h, 24h)

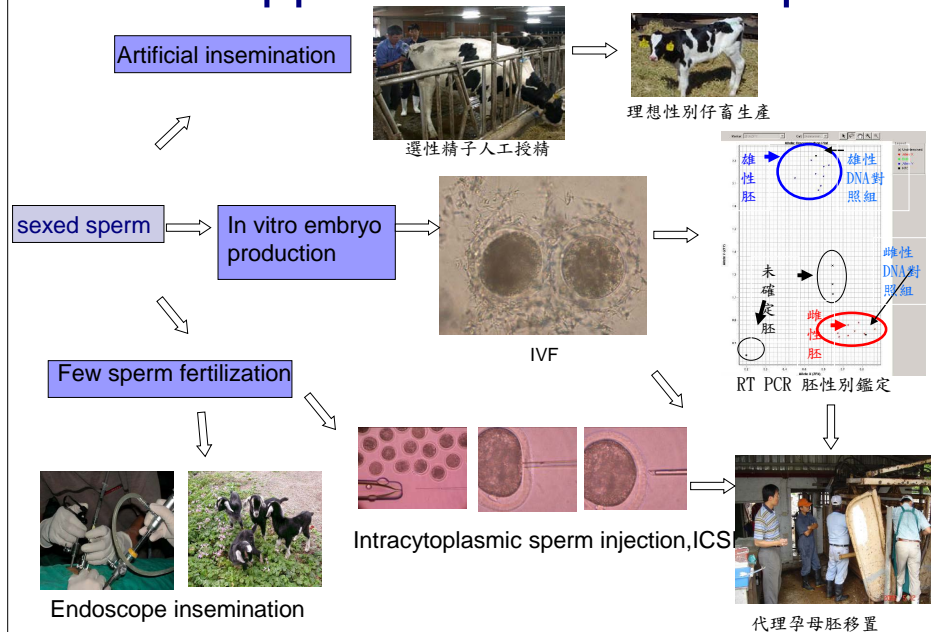
Before the sperm sex separation, 5% LDL Has the protection function

LDL濃度冷藏時間對精子活力之影響

Sperm activity

Y-axis: 活力 (0, 20, 40, 60, 80, 100)
X-axis: 0%, 5%, 6%, 7%, 8%, 9%, 10%

LDL濃度 LDL Concentration

Legend:
- Motility 0h
- Motility 6h
- Motility 24h

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

雄性DNA對照組
雄性胚
雌性胚
雌性DNA對照組
未確定胚

RT PCR 胚性別鑑定

Few sperm fertilization

Intracytoplasmic sperm injection,ICSI

Endoscope insemination

代理孕母胚移置
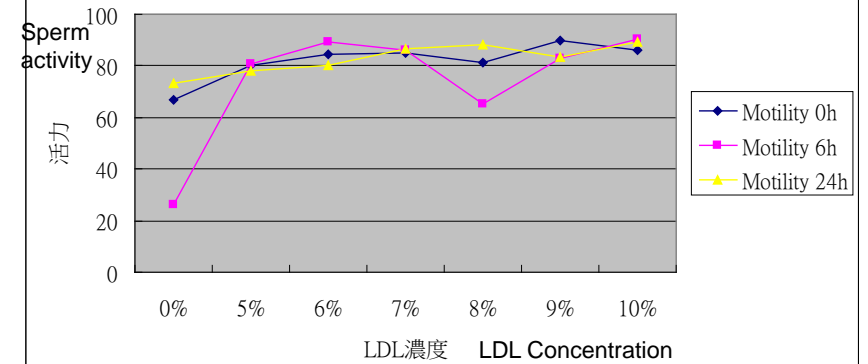
Thanks for Your Attention