17th Harwell Embryo and Spermatozoa Cryopreservation Training Course

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FESA (Frozen Embryo & Sperm Archive)
Medical Research Council
Harwell, UK

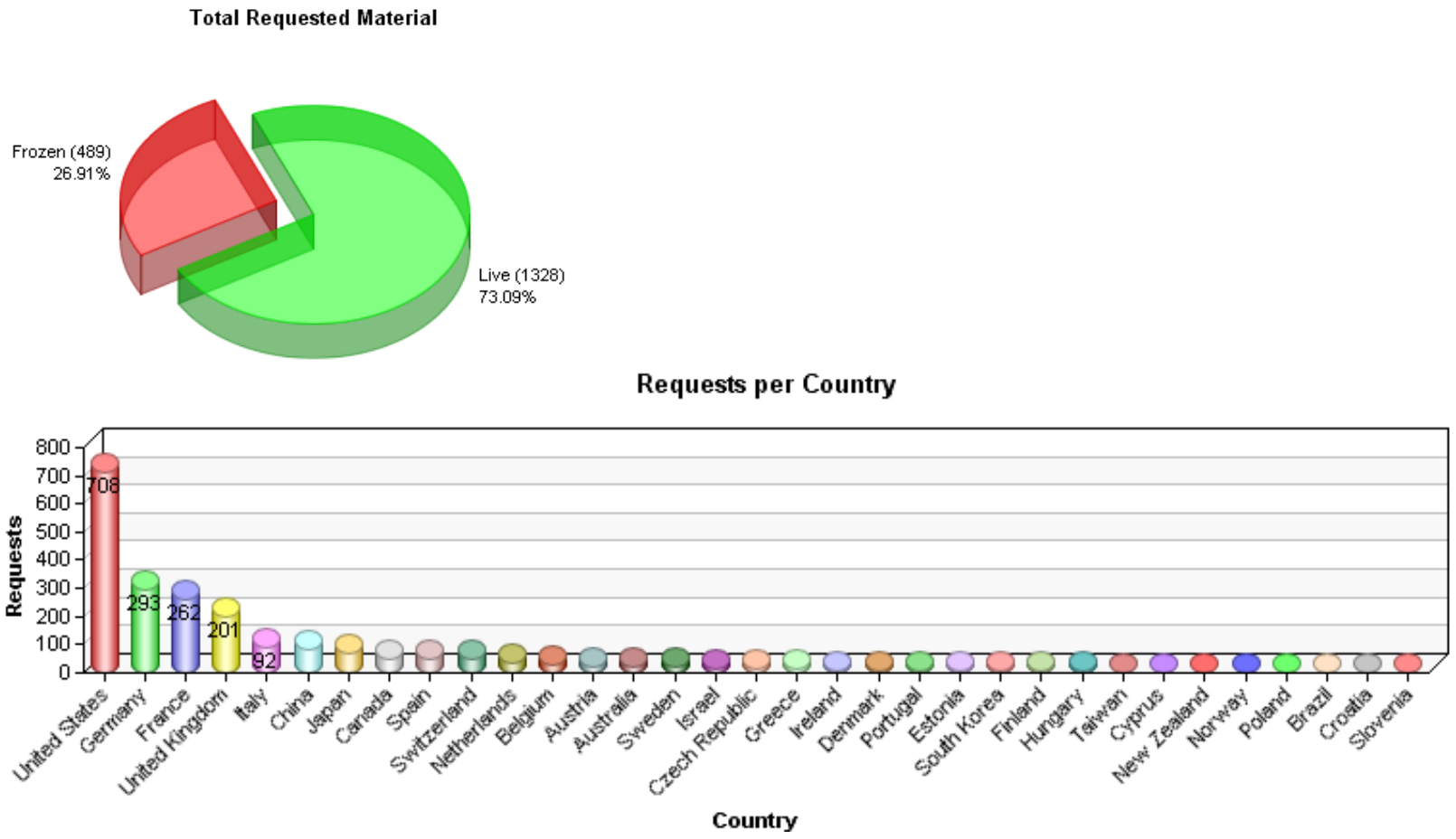
The MRC frozen embryo archive

- Worldwide Genetic Resource
  - ~1450 stocks, >500,000 embryos
    - Includes transgenic, mutants, chromosome anomalies & inbred strains
    - Plus sperm from ~25,000 male mice
- Sole UK archiving centre
- http://www.har.mrc.ac.uk
- **EMMA (European Mouse Mutant Archive)**
- **IMSR (International Mouse Strain Resource)**
- **FIMRe (Federation of International Mouse Resources)**

European Mouse Mutant Archive - EMMA

CNR/IBC
Istituto di Biologia Cellulare, Monterotondo, Italy
CNRS/CDTA
Centre de Distribution, de Typage et d'Archivage animal, Orléans, France
MRC/MGU
Mammalian Genetics Unit, Harwell, UK
Karolinska Institutet
Karolinska Institutet, Stockholm, Sweden
FCG/IGC
Instituto Gulbenkian de Ciência , Oeiras, Portugal
HMGU/IEG
Institute of Experimental Genetics, Munich, Germany
EMBL/EBI
European Bioinformatics Institute, Hinxton, UK
GIE-CERBM/ICS
Institut Clinique de la Souris, Illkirch/Strasbourg, France
Sanger Institute
Wellcome Trust Sanger Institute, Hinxton, UK
CNB/CSIC
Centro Nacional de Biotecnología, Madrid, Spain
Fleming
Biomedical Sciences Research Centre Al. Fleming, Athens, Greece
OULU
University of Oulu, Oulu, Finland
BIAT
Vetmeduni Vienna, Biomodels Austria, Vienna, Austria
IMG
Institute of Molecular Genetics, Prague, Czech Republic

An international service



Mary Lyon Centre – high barrier unit

Course aims

- Hands on demonstration of:
- Embryo freezing
- Sperm freezing
- *In vitro* fertilization

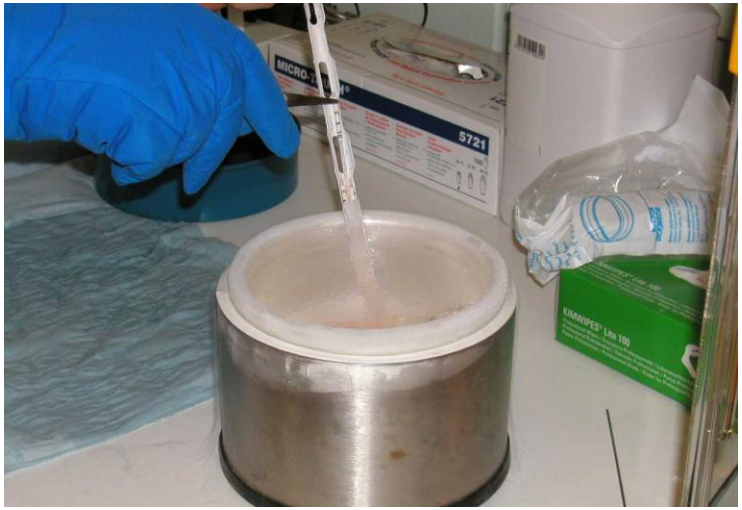
- Reference point
- Disseminate skills

Handling liquid nitrogen

- Asphyxiation – use oxygen monitors
- Colourless, odourless, tasteless gas – no warning
- At low temperatures density is greater than 1
- Cold burns (-196°C) – wear gloves and goggles
- Can condense oxygen from air

What can be cryopreserved?

- Pre-implantation embryos
- Oocytes
- Spermatozoa
- Ovarian tissue



Benefits of cryopreservation

- Reduce number of GA mice on the shelf
- Safety from disease, fire, genetic contamination and breeding failure
- Larger range of stocks available
- Easy disease-free exchange of stocks, nationally and internationally
- Economy
- Stocks remain viable indefinitely

Safe storage

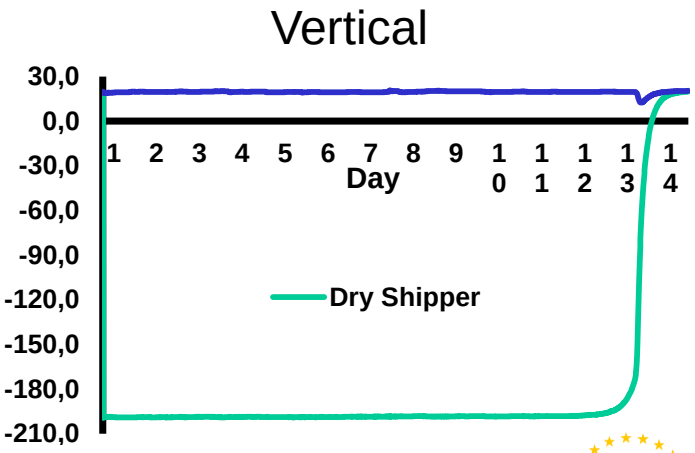
- Glass transition Temp (Tg) = -130°C
- Less than -150°C = no thermodynamic reactions

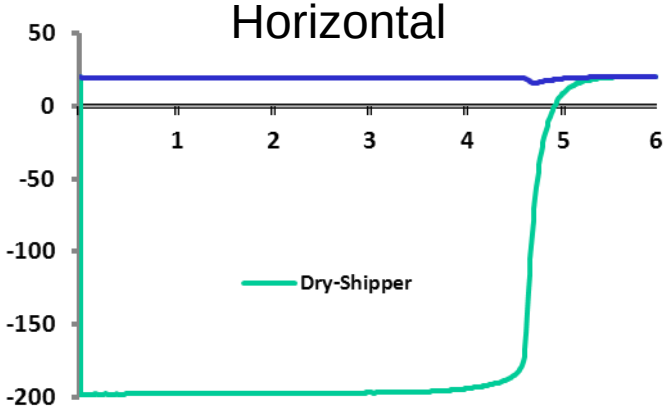
Data management

- Accurate records for data retrieval
  - Stock details
  - Sample id
  - Contents of each cryovial/straw
  - Sample location
  - Freeze/thaw protocol
  - Parental genotype

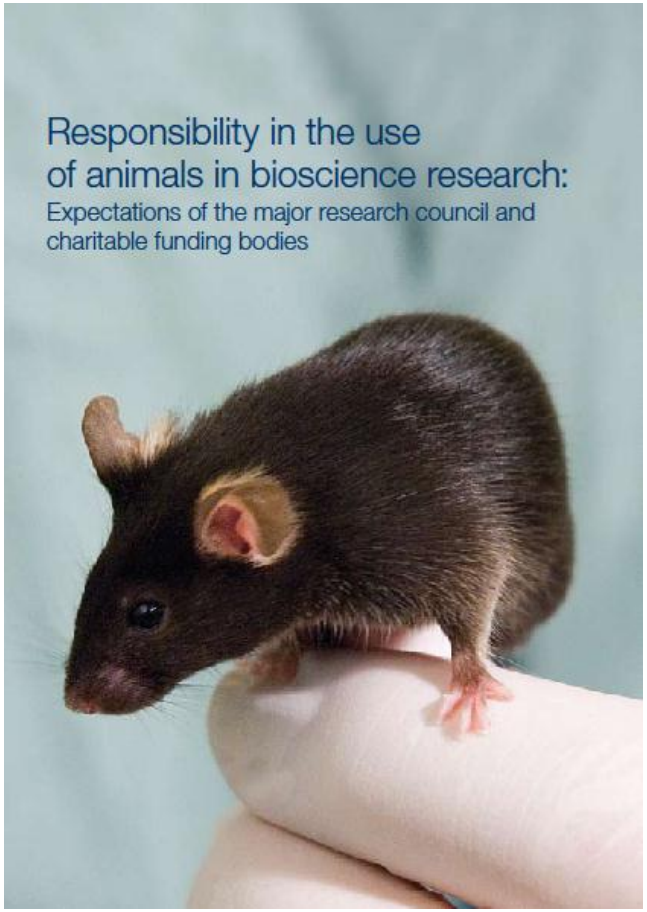
Transport - Dry shipper

- Keep samples at LN_2 Temp
- Re-usable
- Considered safe by IATA
- Robust





Mouse information sheets



MRC | The Mary Lyon Centre

Date 10th June 2010

Dear [redacted]

Mouse Identification and Information Stock code RED/1276 Full nomenclature 129-*Epc1*$^{tm1a(EUCOMM)Wtsi}$/WtsiH

Animal Identifier	Ear Punch	Date of Birth	Sex	Coat Colour	Genotype	Box No	Compartment No
RED/1276.2f	30	21 February 2010	Female	agouti	wild type	Box 1 Compartment 1	
RED/1276.2h	10	21 February 2010	Female	agouti	Heterozygous		
RED/1276.2g	3	21 February 2010	Female	agouti	wild type		
RED/1276.2i	30	21 February 2010	Male	agouti	Heterozygous	Box 1 Compartment 2	
RED/1276.2j	3	21 February 2010	Male	agouti	Heterozygous		
RED/1276.1h	10 + 33	21 February 2010	Male	agouti	wild type		

Other Information

Background and origin of strain: 129S5/SvEv. Derived from the Sanger ES cell resource

Immune compromised: No / Yes (reason) no

Coat Colour: agouti (wild-type)

Phenotype: no visible phenotype

Husbandry conditions (current diet / bedding / substrate / environmental enrichment): diet –SDS RM3 (E) –expanded diet; bedding –Datesand grade 5 wood shavings; substrate –'Transgel'- water/food supplement for shipment; environmental enrichment – funnel tunnels, paper bedding.

Breeding recommendations: normal breeding regime x 129S5/SvEv

Other comments: Please Note that RED is our in house stock name

Landmarks in cryopreservation: 1

- 1949: Parkes, Smith & Polge
  - Demonstrated cryoprotective properties of glycerol on fowl sperm
- 1952: Audrey Smith
  - Rabbit granulosa cells grown in culture after freezing (-79^0 C) in 15% glycerol
- 1953: Parkes & Smith
  - Showed that rat ovarian tissue retained some endocrine activity after freezing in 15% glycerol

Landmarks in cryopreservation: 2

- 1956: Alan Parkes
  - Demonstrated that frozen mouse ovarian tissue retained viability after grafting
- 1960: Delphine Parrott
  - Froze mouse ovarian tissue in 15% glycerol in horse serum to -79^0 C
  - Obtained live mice after orthotopic transplantation of the thawed tissue
  - **First incidence of live mice from cryopreserved materials**

Landmarks in cryopreservation: 3

- 1971: David Whittingham
  - Reported live mice born from embryos frozen to -79 ^{0}C in PBS + 7.5% PVP
- 1972: Ian Wilmut
  - Could not repeat the above, but got survival of mouse embryos frozen in 1.5M DMSO in LN_2
- 1972: Whittingham, Leibo & Mazur
  - **Many live mice from embryos frozen in 1M DMSO in LN_2**

Landmarks in cryopreservation: 4

- 1974: David Whittingham
  - “Embryo Banks in the Future of Developmental Genetics” Genetics 78
- 1974: Lyon, Whittingham & Glenister
  - Began feasibility studies on long-term storage of mouse embryos of various genotypes

Stability of the mouse genome

- Embryos stored under low-dose γ irradiation to simulate long-term storage
  - No effect of irradiation found on:
    - Morphological appearance after thawing
    - Survival to blastocyst after overnight culture
    - Survival of foetuses and live-born after transfer
    - Offspring bred normally and showed no evidence of genetic defects
  - 200cGy - Simulated storage of up to 2000 yr. under normal levels of background radiation

Recovery of genetic variants

- Various mouse stocks recovered after embryo cryopreservation:
  - Inbred strain (CBA/CaH)
  - Inbred strain + translocation (CBA/H-T6)
  - Dominant sex-linked gene (*Mo^{dp}*)
  - Multiple recessive stocks:
    - PT (*aa bb c^{ch}c^{ch} dd pp ss sese*)
    - HT (*aa bpbp fzfz lnln papa pepe*)
  - XO (tagged with *Ta* & *Mo^{blo}*)

Brief history of mouse sperm cryopreservation

Sperm fertilisation ability is influenced by plasma membrane reorganisation, cholesterol sequestration, intracellular Ca^{++} and reactive oxygen species.

1992: Nakagata & Takeshima – 3% raffinose & 18% skimmed milk

2007: Takeo – 0.75mM methyl β-cyclodextrin (MBCD), plus 1mg/ml PVA added to post thaw media

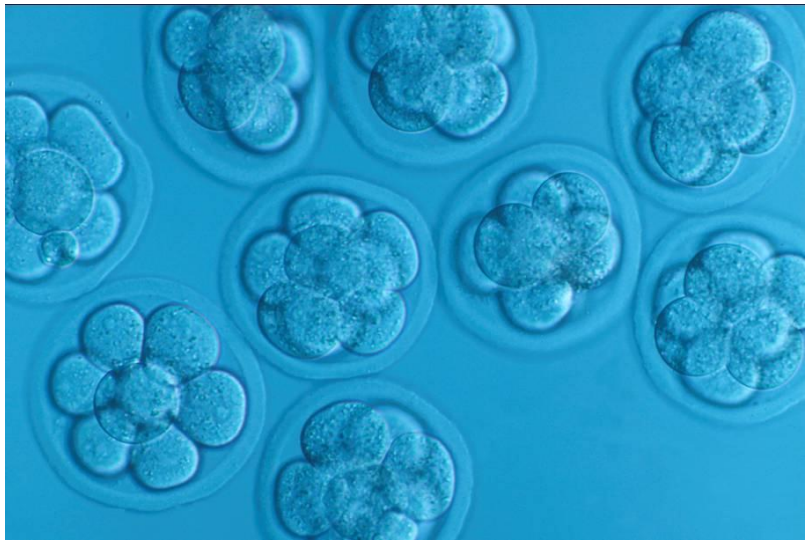
2008: Ostermeier - 477 µM Mono-thioglycerol (MTG) added to CPA

2009: Liu – AA included in CPA, plus methyl β-cyclodextrin in post thawing media

2010: Bath - removal of inhibitory factors, plus reduced glutathione in IVF media

2011: Takeo – MBCD treatment, plus reduced glutathione in IVF media

Embryo freezing at Harwell

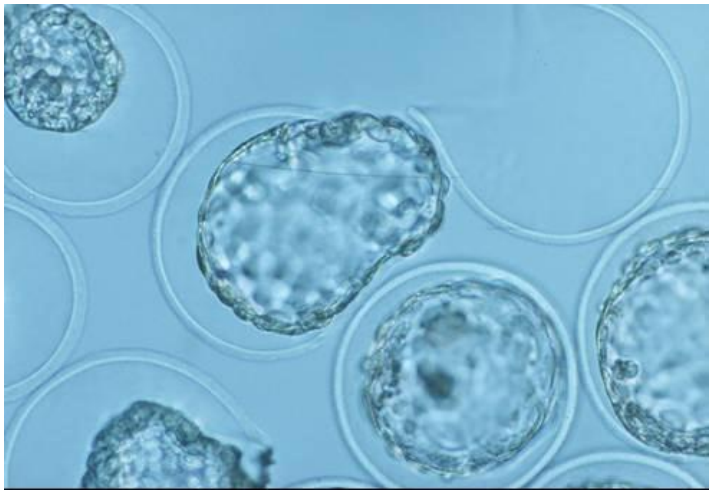


Cryopreservation of the pre-implantation embryo

- Controlled rate freezing
- Vitrification

Key aspects of cryopreservation

- Cryoprotectant used
- Seeding temperature
- Freezing rate
- Thawing rate



Types of cryoprotectants

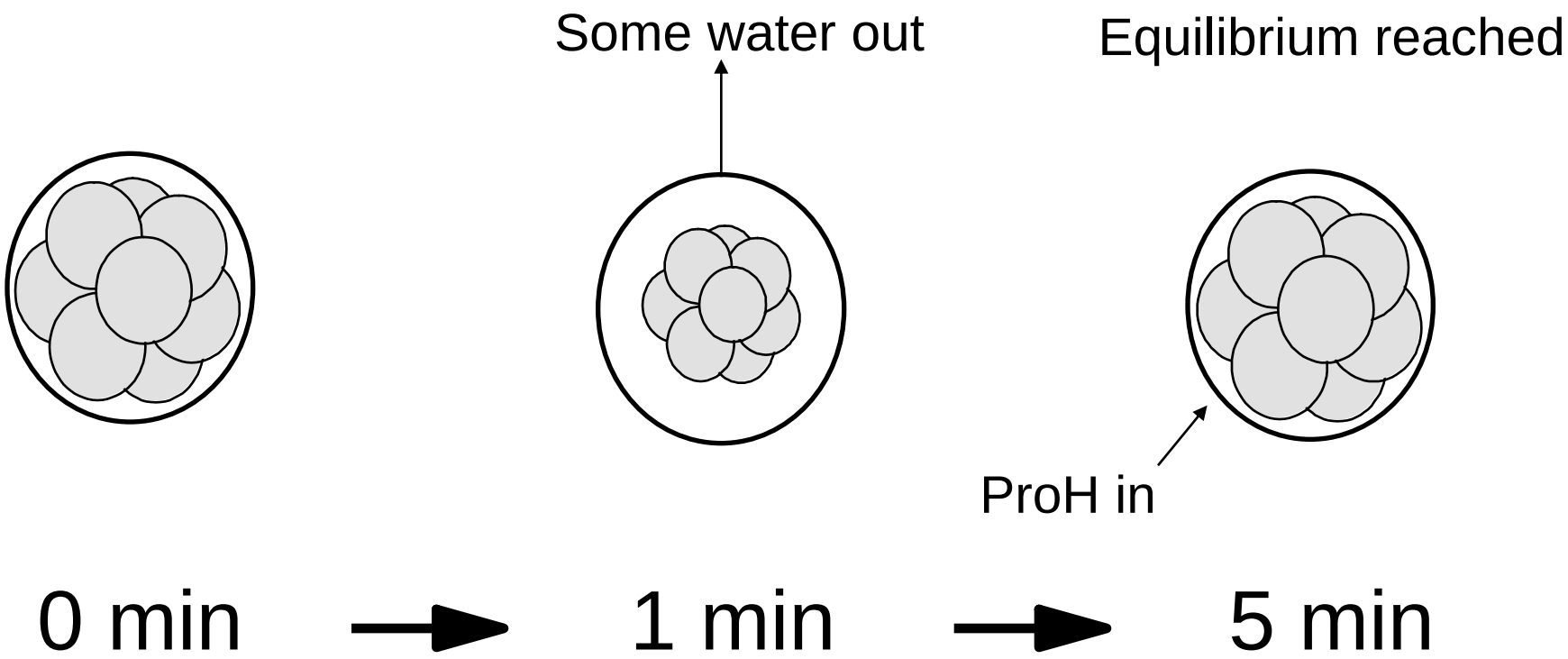
- Alcohols (ethylene glycol, propylene glycol)
- Amines (formamide, taurine, lysine, proline)
- Inorganic salts (ammonium sulphate)
- Macromolecules (skim milk, serum, PVP, PEG)
- Sugars (sucrose, maltose, raffinose, trehalose)
- Dimethylsulphoxide

Embryo cryopreservation: protocol

- Cryoprotectant and diluent:
  - 1.5M Propylene Glycol in Medium M2
  - 1.0M Sucrose in Medium M2

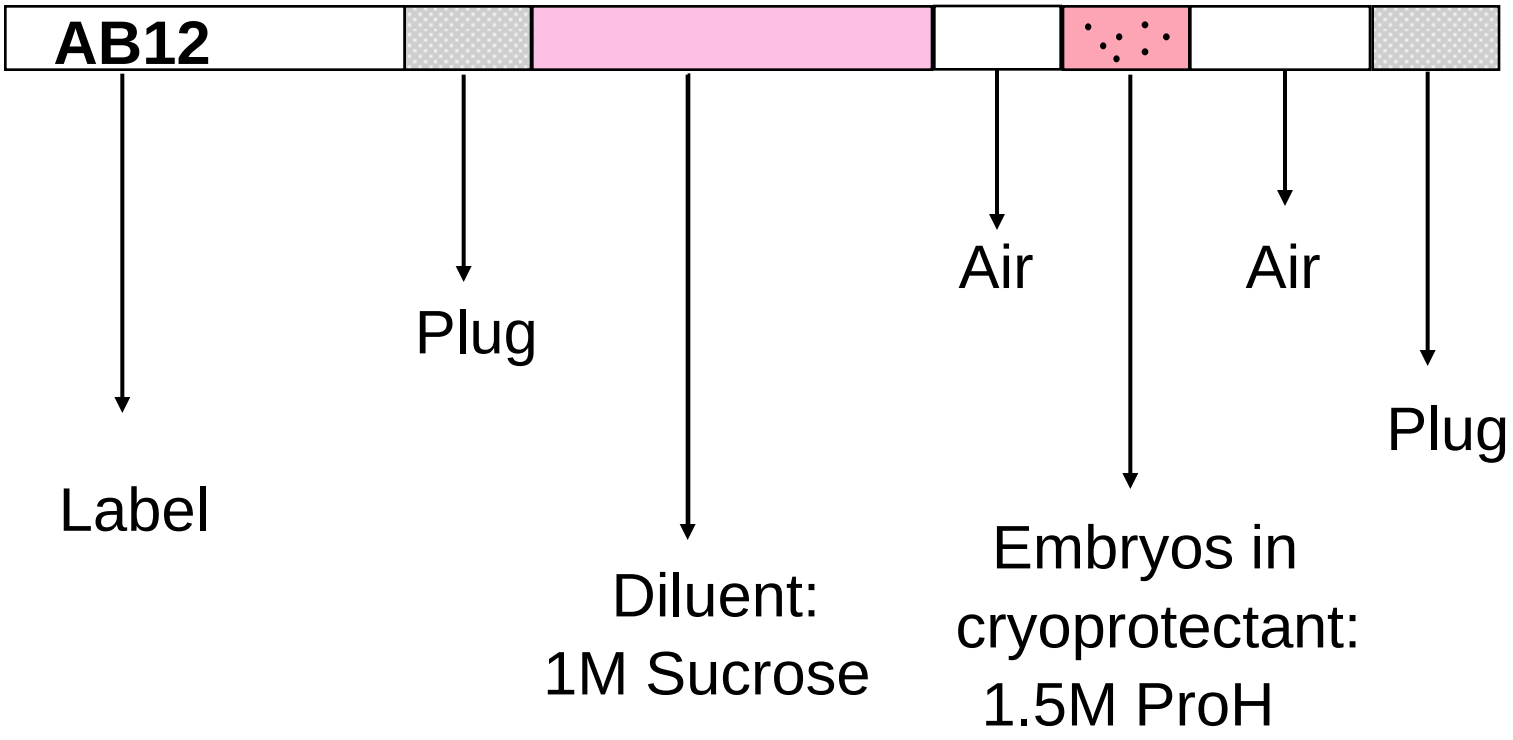
- Embryos frozen in plastic semen straws
  - Protocol of Renard & Babinet, 1984

8-cell embryo in 1.5M ProH

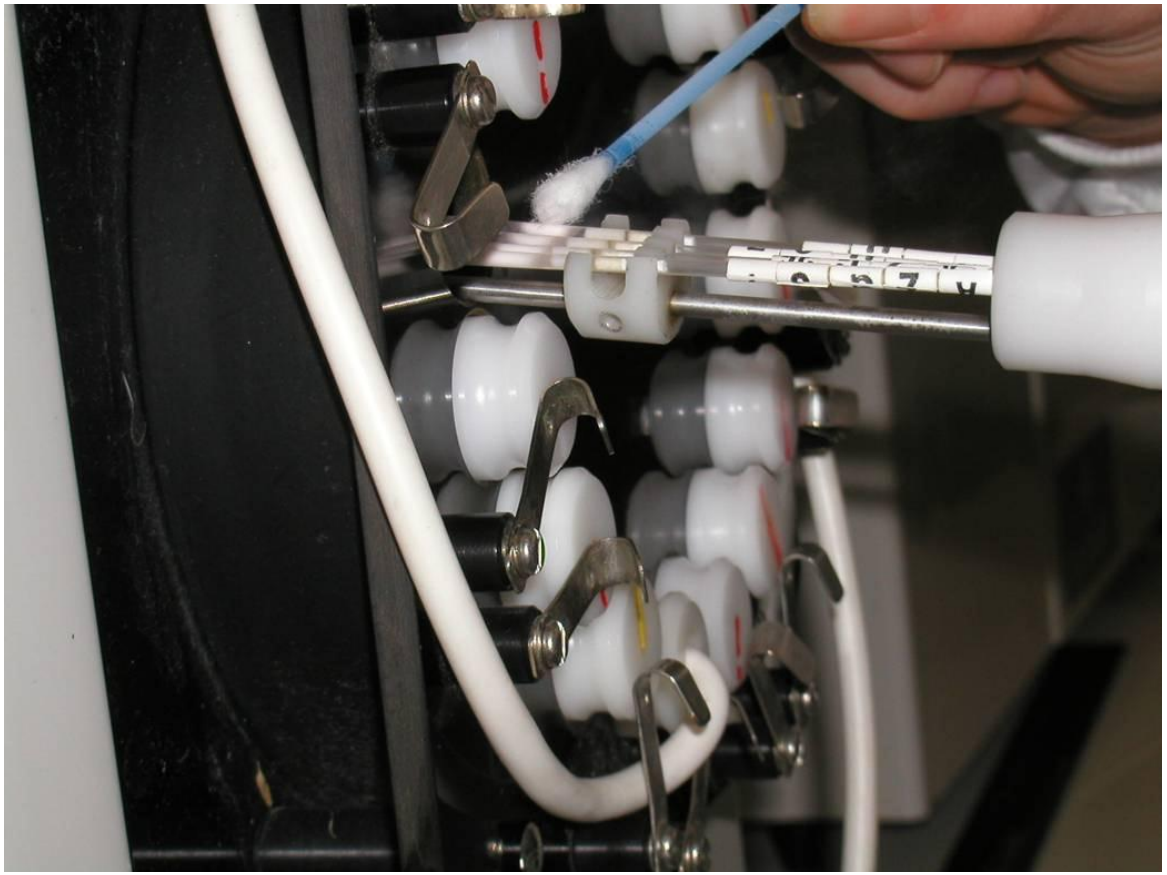


Loading embryos

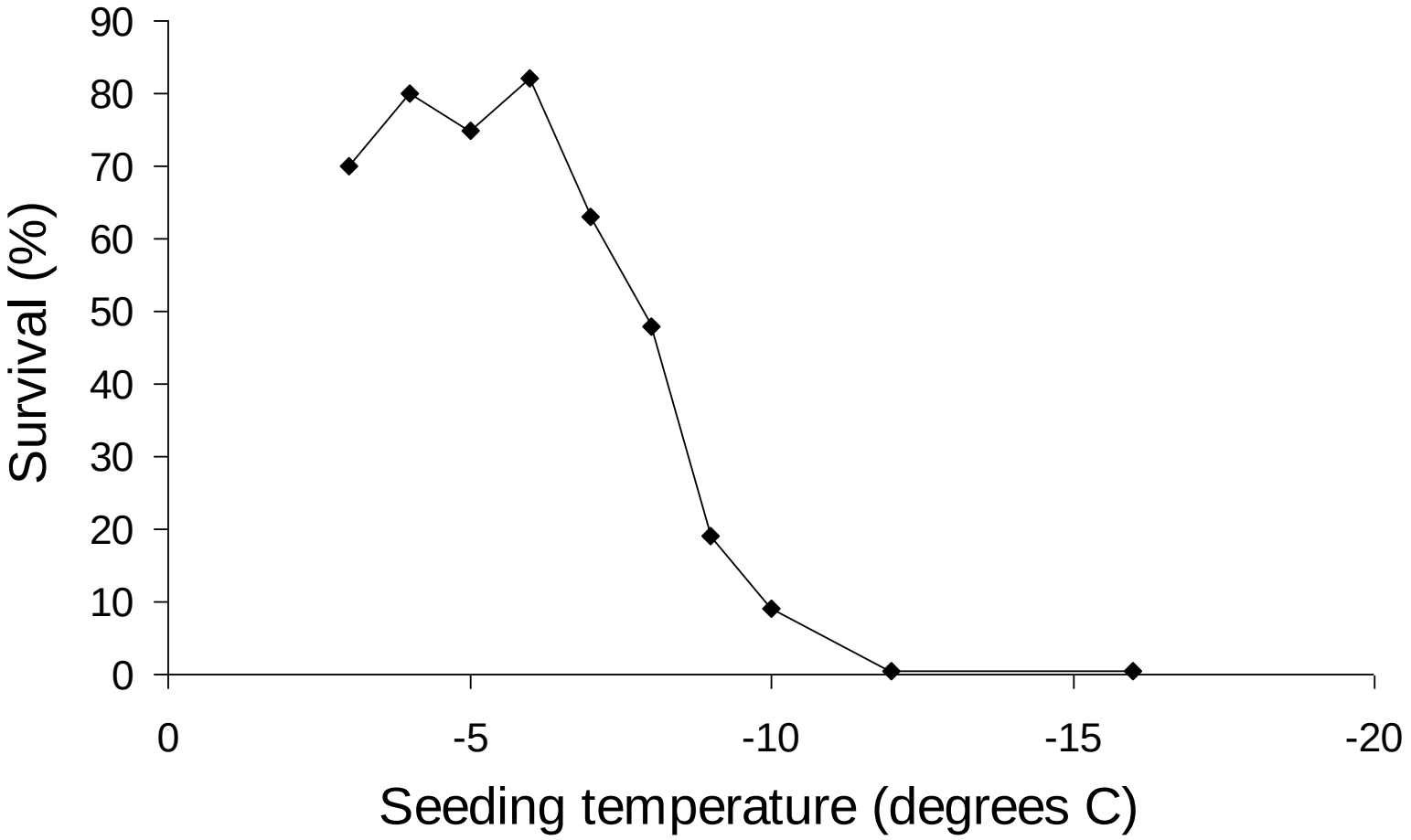
Embryos frozen in plastic semen straws



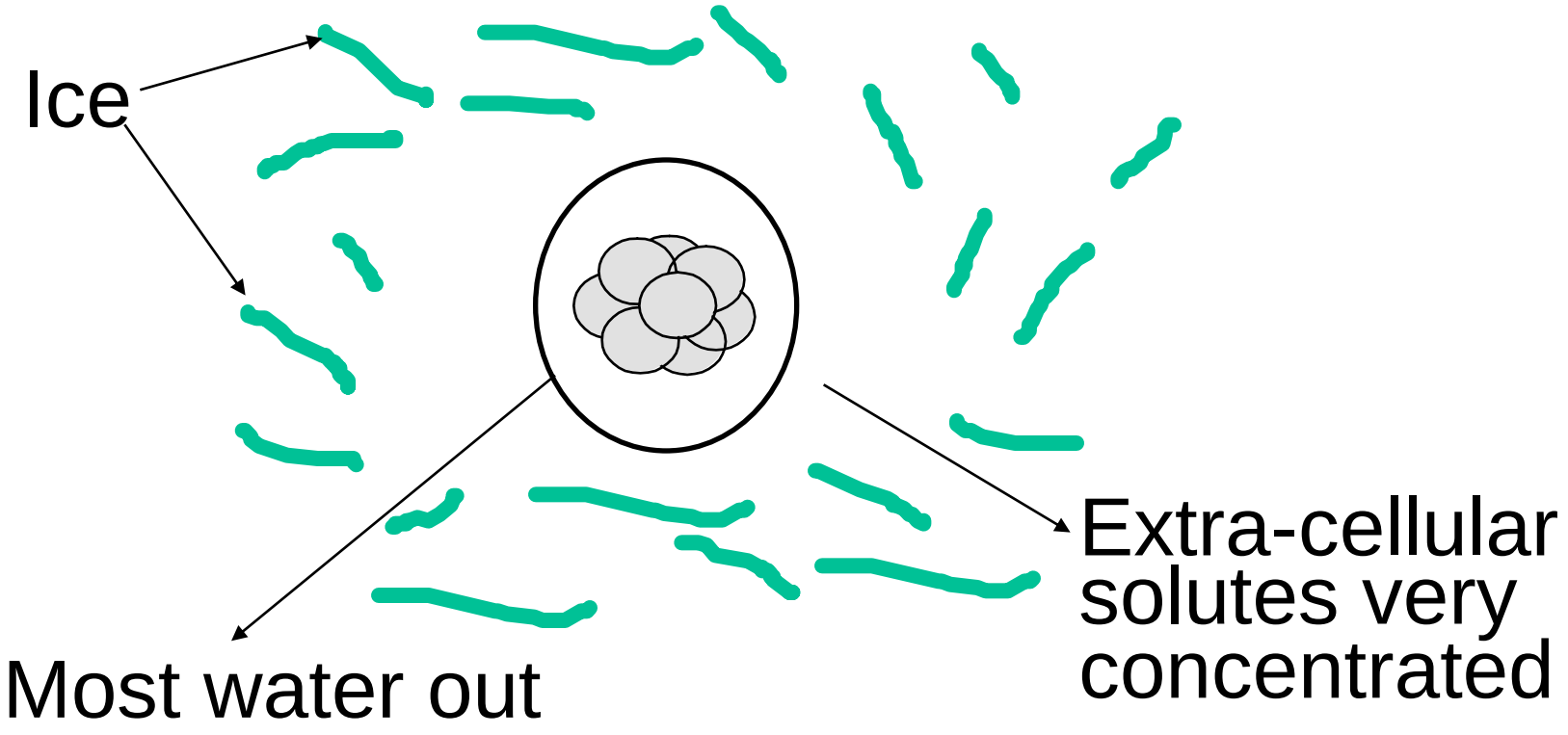
Seeding the straws



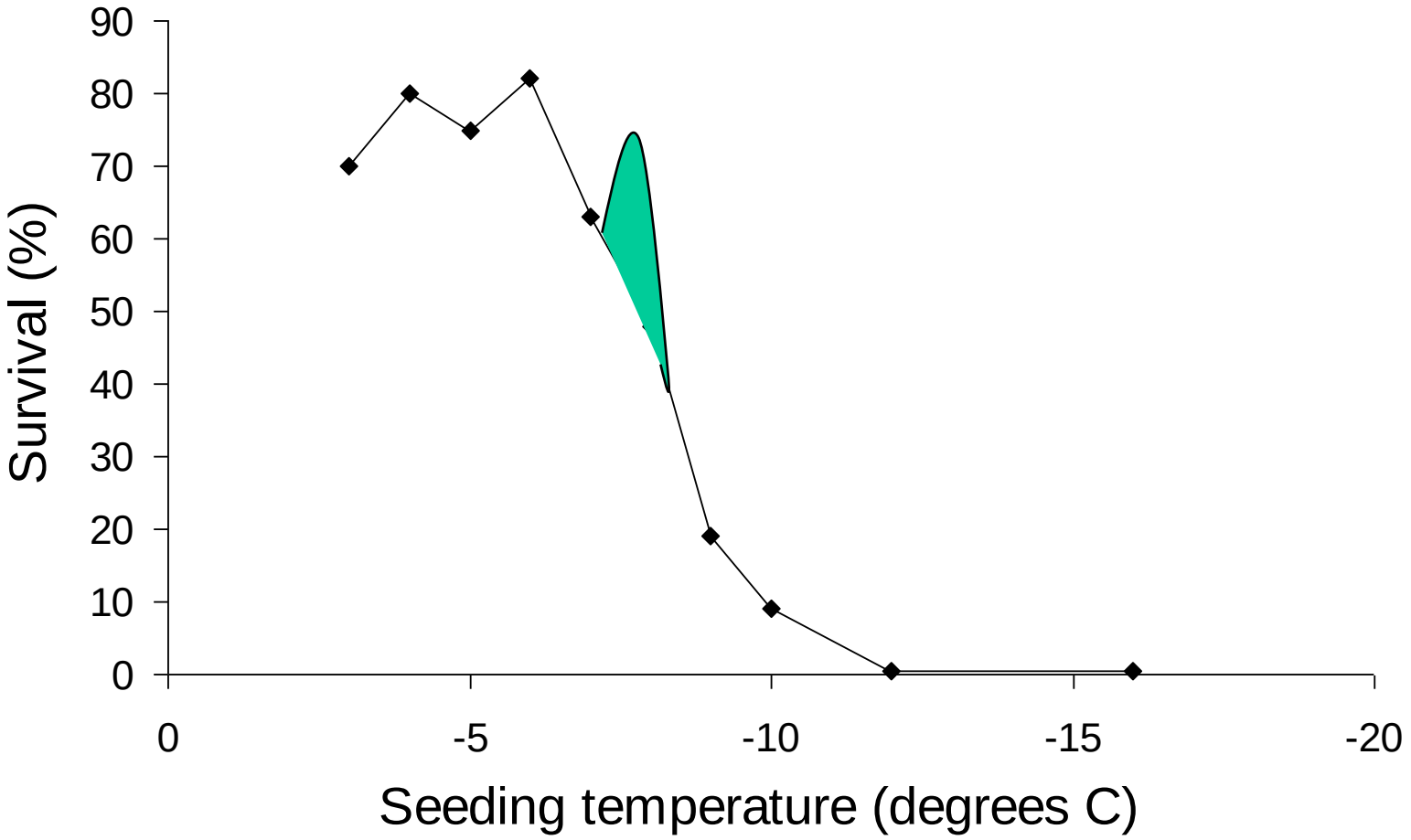
Effect of seeding temperature



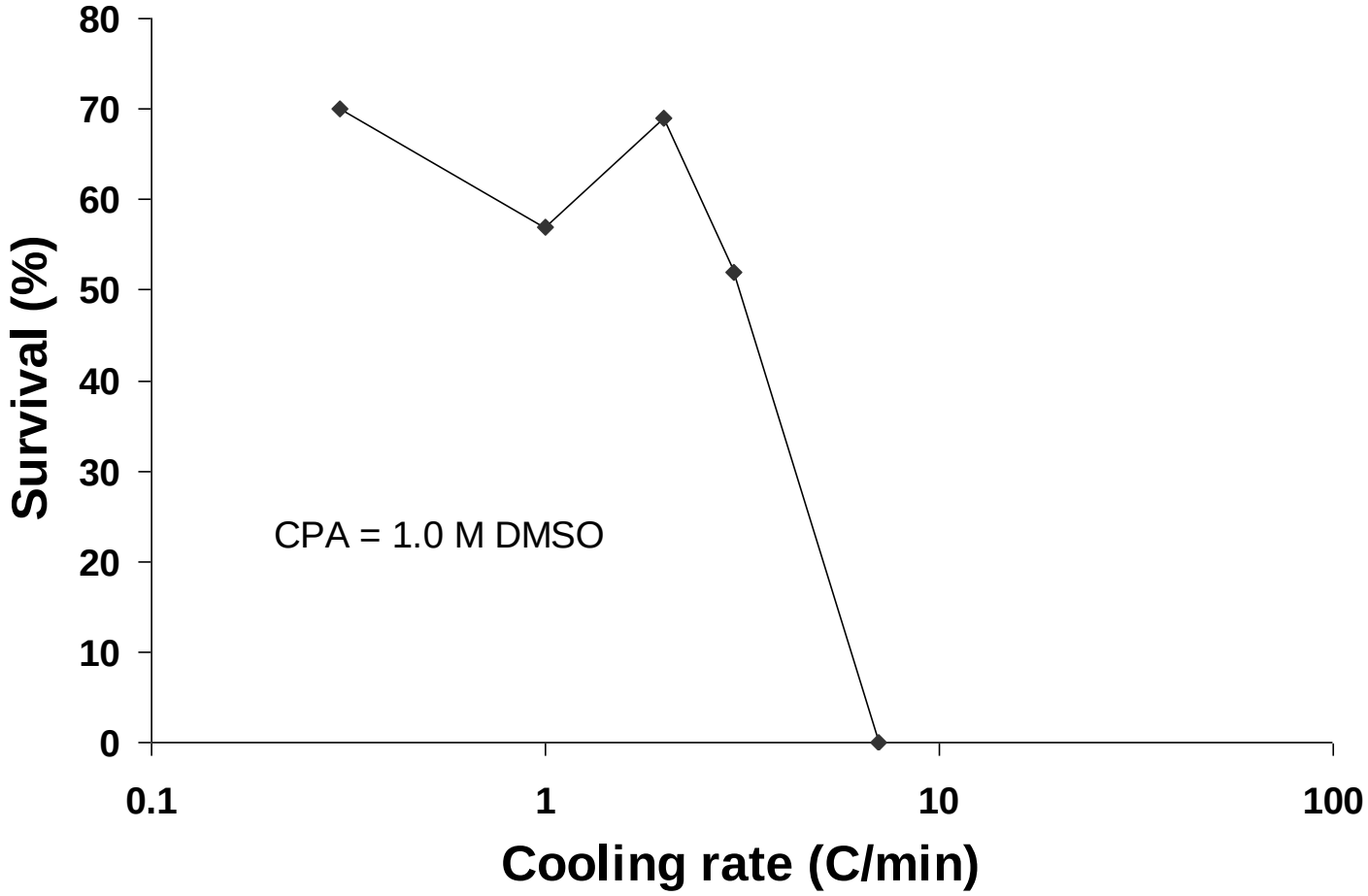
8-cell embryos cooled to -30^{0}C at 0.3 to 0.5^{0}C/min.



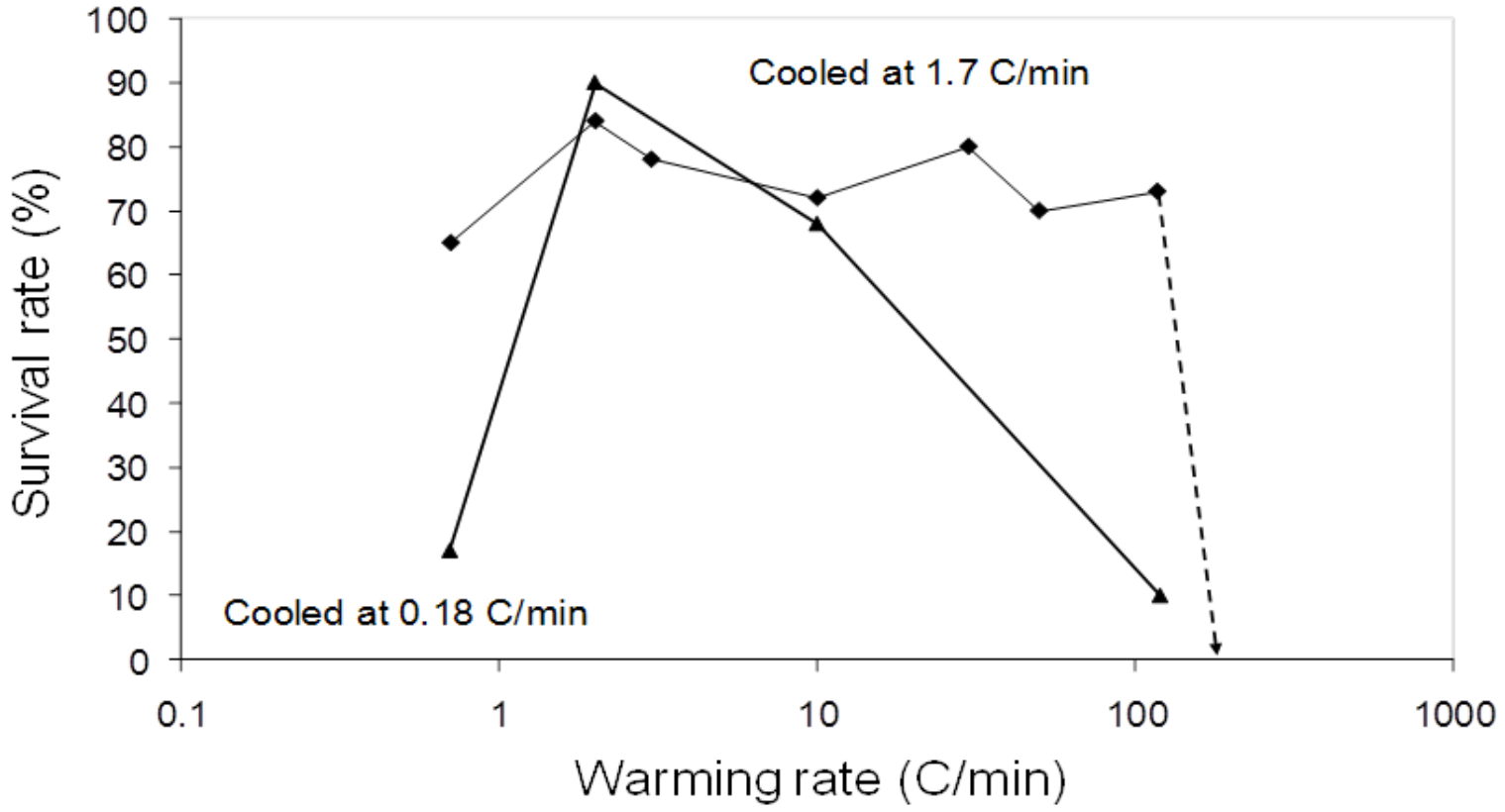
Effect of seeding temperature



Effect of cooling rate - Whittingham *et al* (1972)

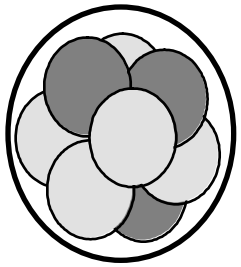


Effect of warming rate - Whittingham *et al* (1972)



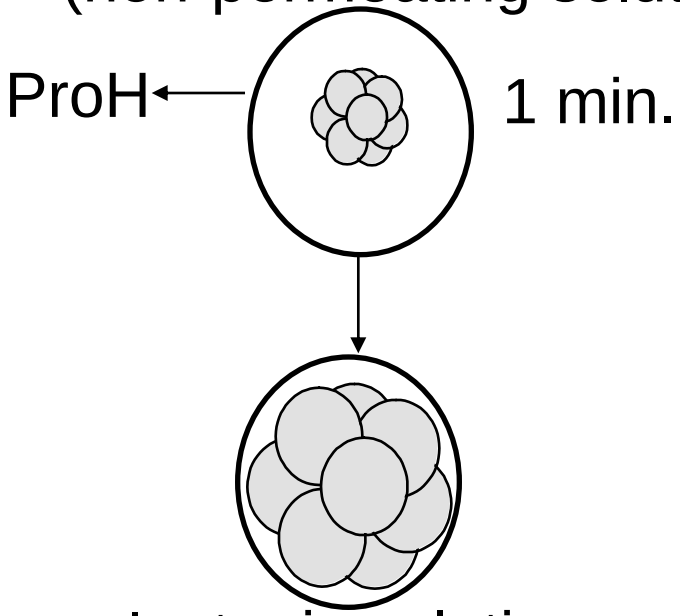
Embryo shortly after rapid warming from -196^{0}C

No Sucrose



Rapidly swollen embryo containing ProH and water (damaged)

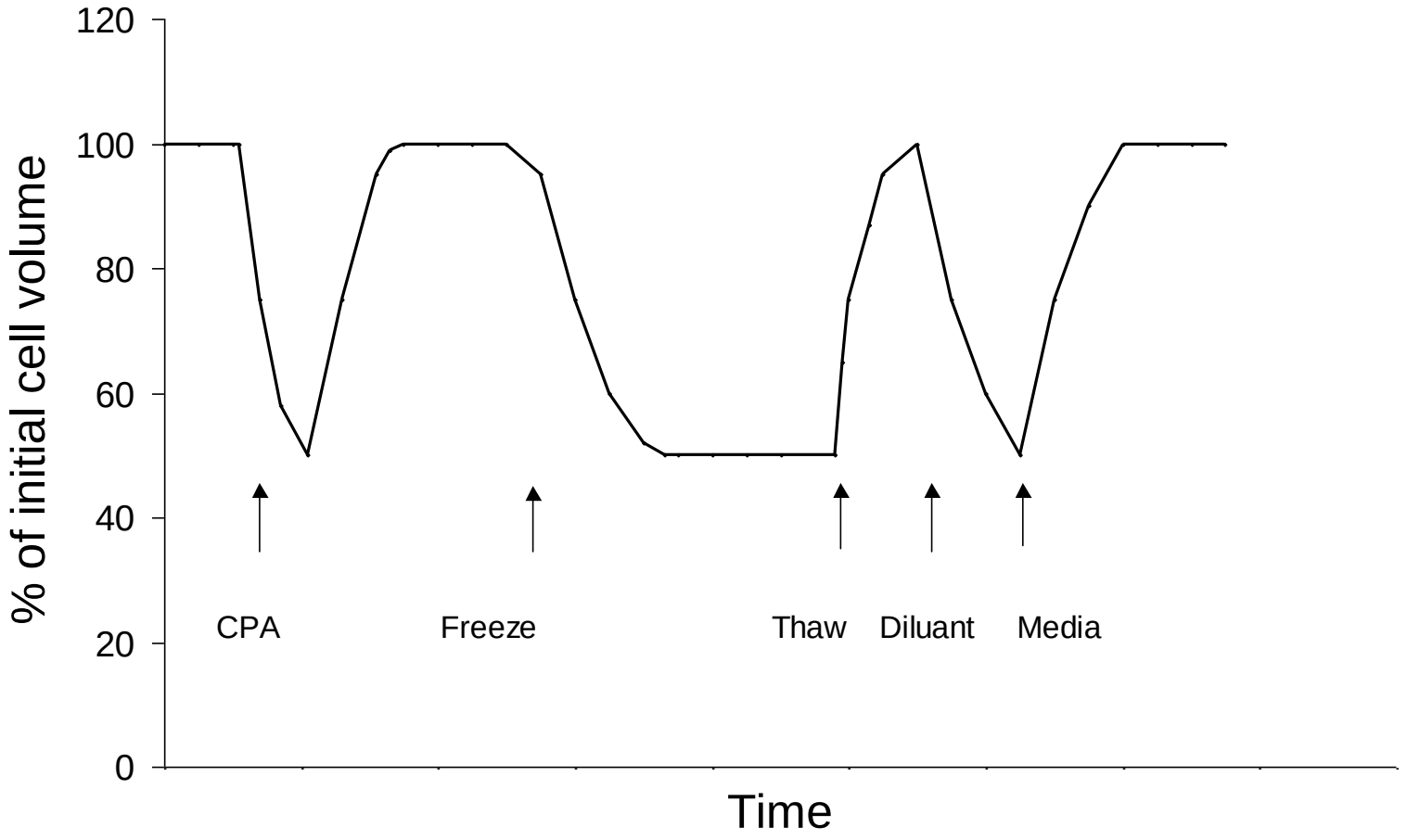
1.0M Sucrose
(non-permeating solute)



Isotonic solution.

5 min.

Embryo freezing dynamics



Embryo vitrification

- Numerous protocols exist in the literature e.g. Nakao et al 1997
- No expensive equipment associated with controlled rate cooling regimes.
- Advantage - embryos are not subject to chilling injury or blastomere damage resulting from intra- or extracellular ice crystal formation.
- Disadvantage – need precise control of the time the embryos are exposed to the vitrification solutions and the temperature of those solutions.
- The cryoprotectant (DAP213) solution used - a mixture of 2M DMSO, 1M Acetamide and 3M Propylene glycol made up in PB1 medium.

Cryopreservation of mammalian sperm

Mammalian Orders of Sperm Cryopreserved

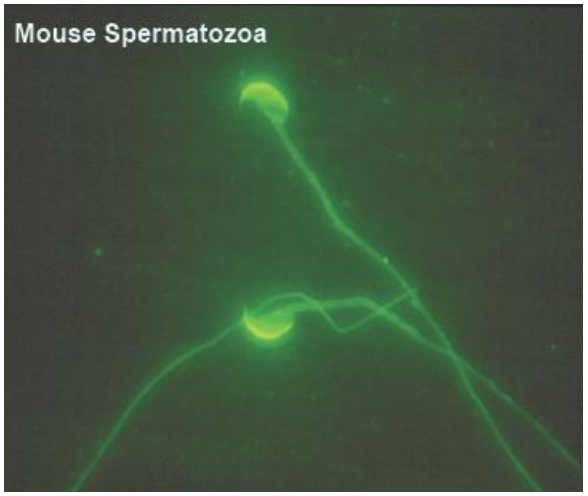
Order	Examples
Artiodactyla	*24 species: bovid, camel, deer*
Carnivora	*14 species: cat, dog, cheetah, fox*
Cetacea	*1 species: dolphin*
Lagomorpha	*1 species: rabbit*
Perissodactyla	*1 species: horse*
Rodentia	*2 species: mouse, rat*
Primates	*7 species: human, gorilla, chimp*

Cryopreservation of mouse sperm

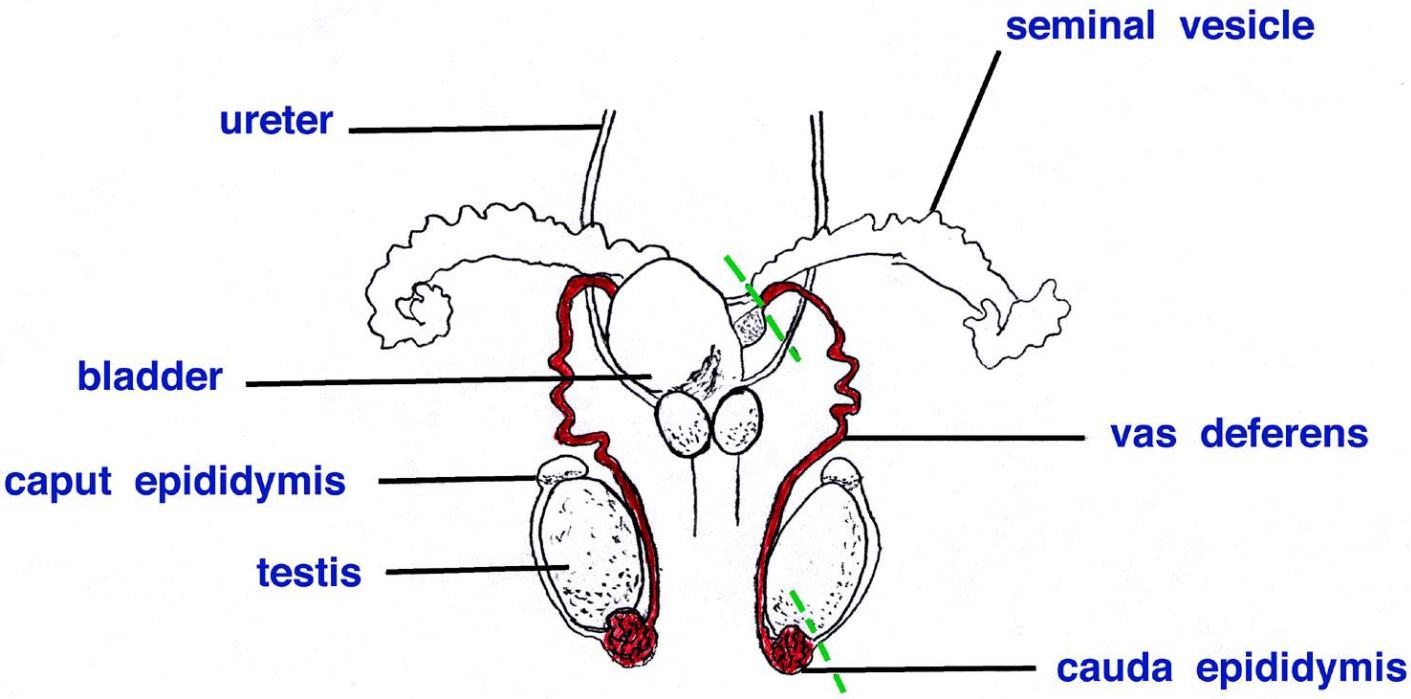
- Low tech in comparison with embryo freezing

Sperm freezing: applications

- Archiving, plus DNA library
- Emergency cryopreservation of sick males
- Export/Import mutants
- Cheap and easy
- Rapidly freeze down stock
- Small number of donors required



Urinogenital system of mouse



Sperm freeze method - 1

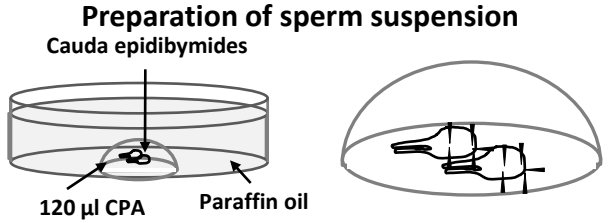
- Dissect cauda epididymides
- gCPA (100mM L-glutamine in 18% raffinose 3% skimmed milk
- Harvest sperm from the cauda
- 3 mins in 120µl gCPA
- Load 10µl sperm into straws
- 10 mins in LN_2
- Plunge in LN_2

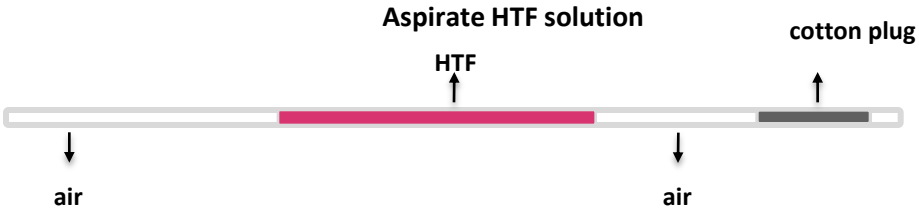
- Thaw sperm in by plunging into 37ºC water bath for 10 mins

Sperm freeze method - 2

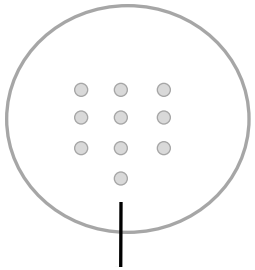
Collection of cauda epididymides

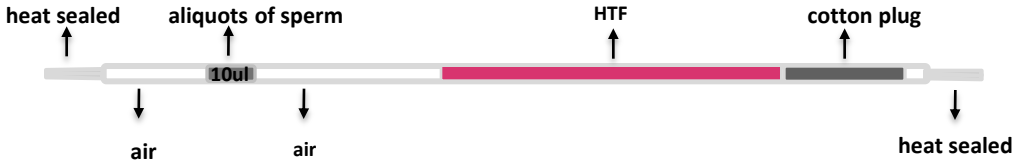
Preparation of sperm suspension



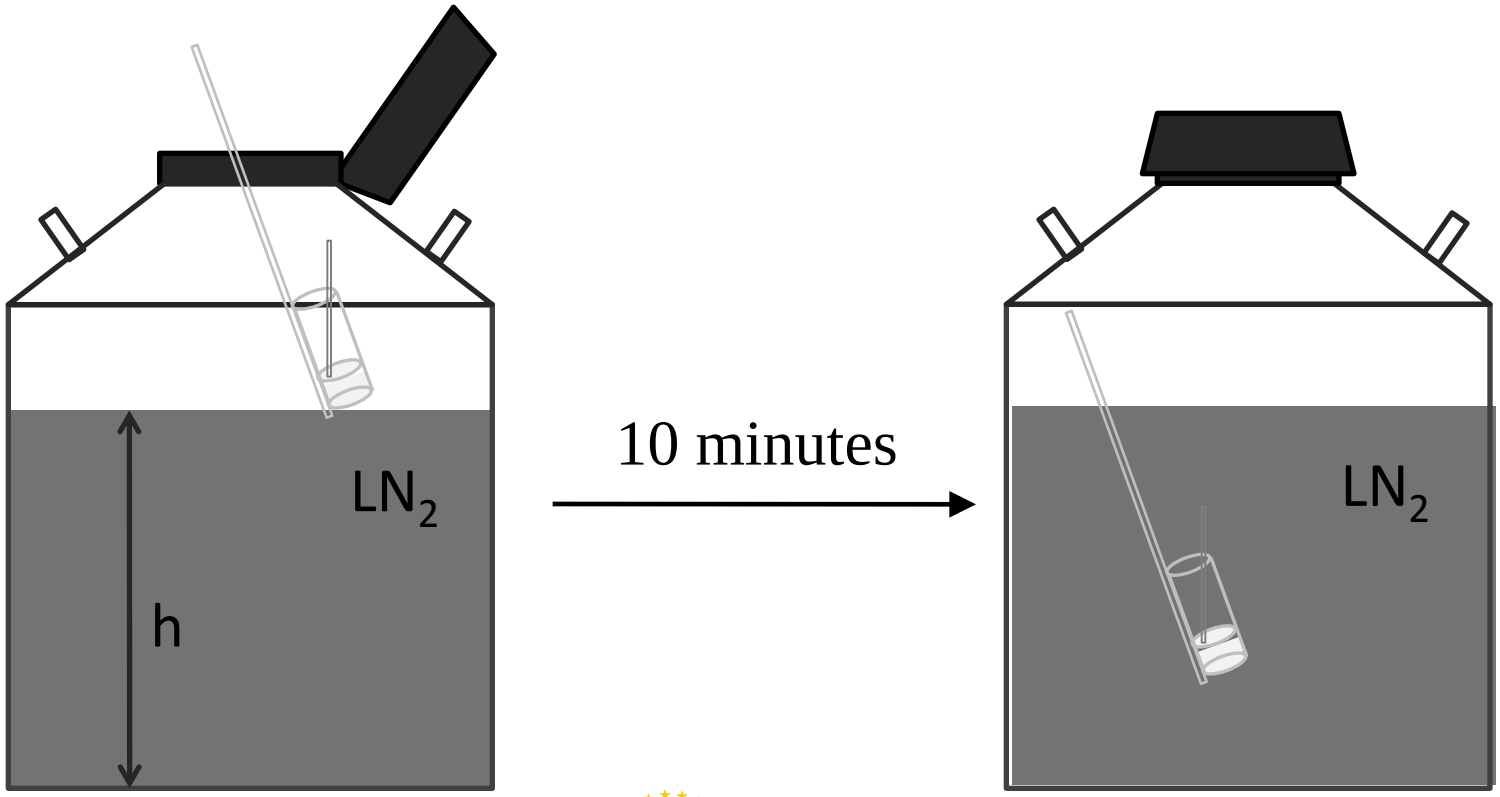


Load the straw

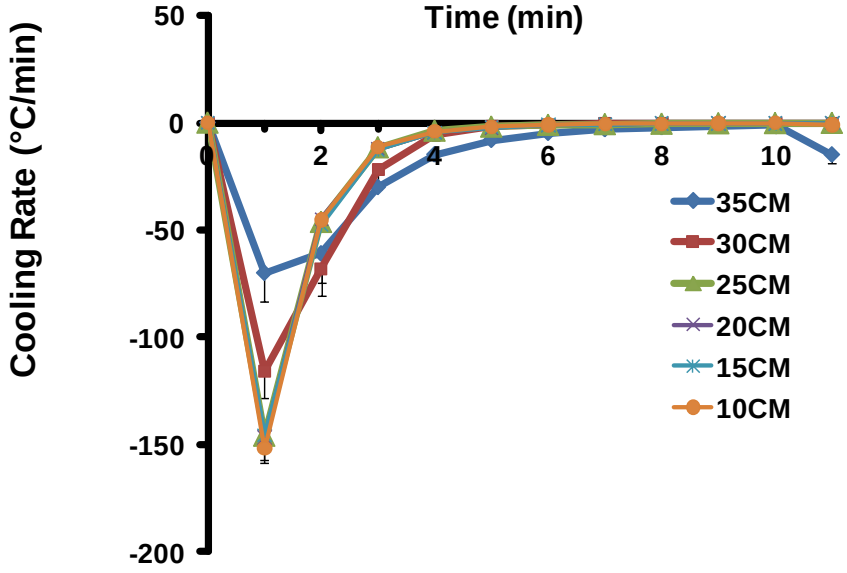


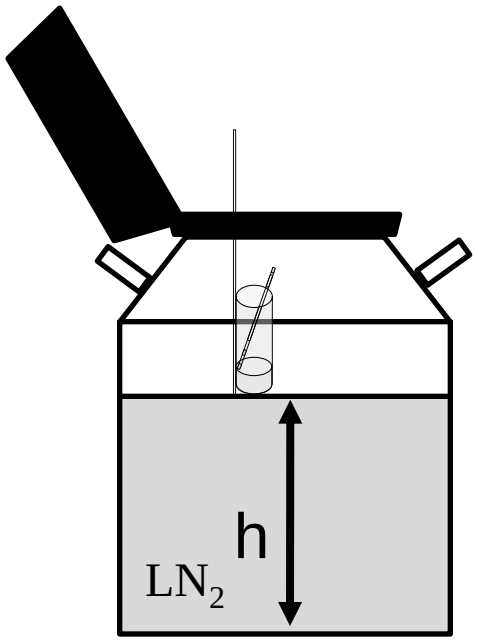


Sperm freeze method - 3



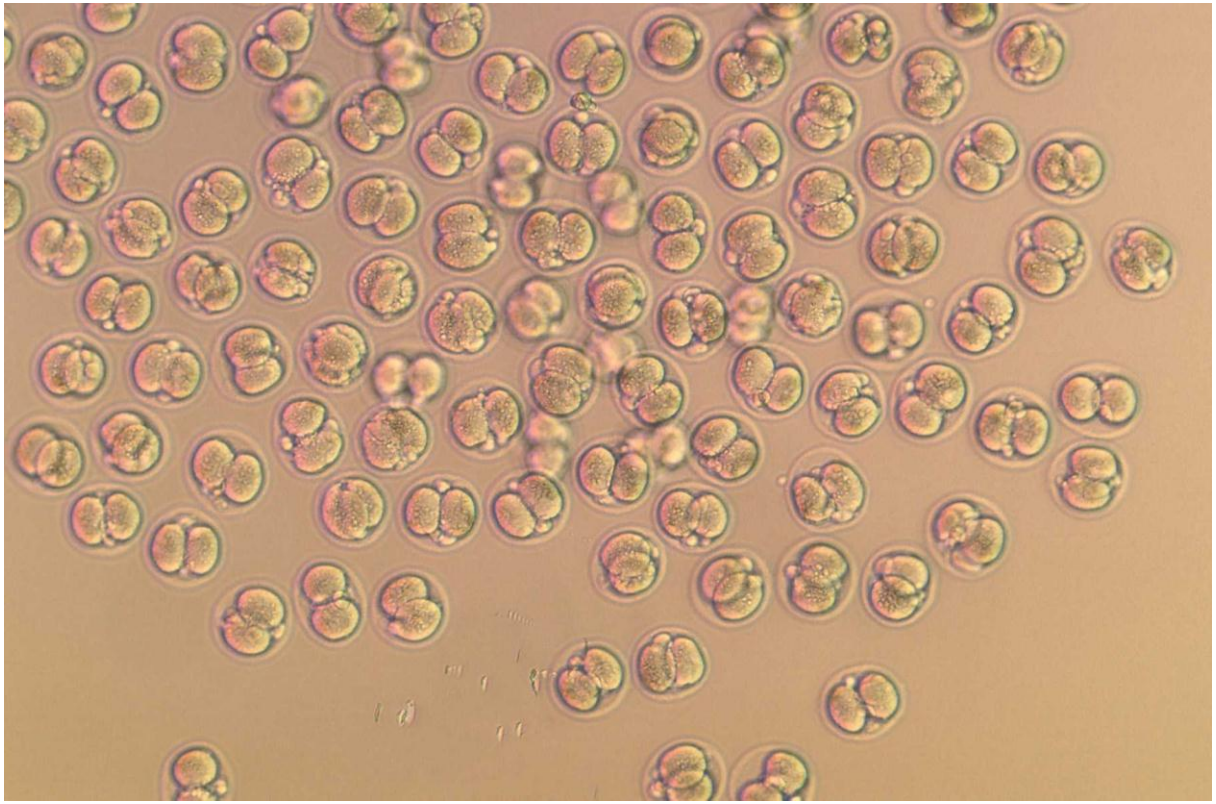
Sperm freezing profile





Groups	Cooling rate (°C/min)	No. of females used	No. of oocytes harvested	No. of embryos used	No. of 2-cells produced	Fertilisation rate (%)
BOX	-37	6	233	223	36	16.14
35cm	-70	6	201	185	44	23.78
30cm	-115	6	166	154	32	20.78
25cm	-145	6	245	203	74	36.45

In vitro fertilization



Exploitation of *in vitro* fertilization

- Rapidly build up new stocks
- Fast-track embryo freezing
- Recovery of mutants
- Colony rescue
- Can achieve >100 offspring per IVF
- Produce cohorts of age matched animals exhibiting age related or progressive phenotype

IVF with frozen/thawed sperm

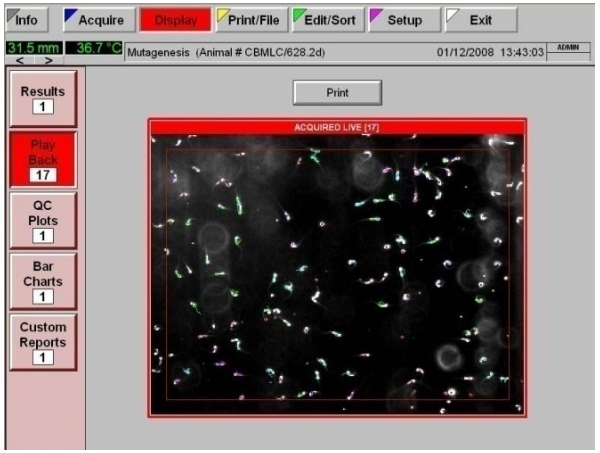
- Warm frozen straw in 37ºC for 10 mins
- Disperse 10µl of sperm into a 90µl drop of TYH + 0.75mM MBCD for 30mins at 37ºC
- Add up to 6 x cumulus masses; ~14 hours post hCG
- Use high Ca^{++} HTF supplemented with 0.25mM GSH
- Incubate for 3 to 5hrs, 37ºC, 5% CO_2 in air
- Wash eggs, culture overnight in 150µl high Ca^{++} HTF
- Transfer 2-cell embryos to oviducts of 0.5 day pseudopregnant recipients

Potential of IVF using frozen sperm – (MBCD method)

- 10 x 10μl aliquots of frozen B6N (Sinann, IVF/2605) sperm used to fertilise 209 x B6N oocytes *in vitro*
- Only 50% of sperm in equilibration drop was used
- 201, 2-cell embryos obtained (97% fertilisation)
- 36 x transferred to 2 recipient females, 23 animals born (63% implantation rate)
- If all frozen sperm was used in similar IVFs, we predict ~2532 offspring from this male

The potential of sperm freezing:

- Theoretically possible to recover >2000 mice from the frozen sperm of one male
- Limiting Factors:
  - No. of eggs available for IVF
  - No. of recipient females
  - Genotype dependent
  - Perform IVF viability tests on stocks
  - CASA
    - Nakagata (2000) Mammalian Genome 11, 572

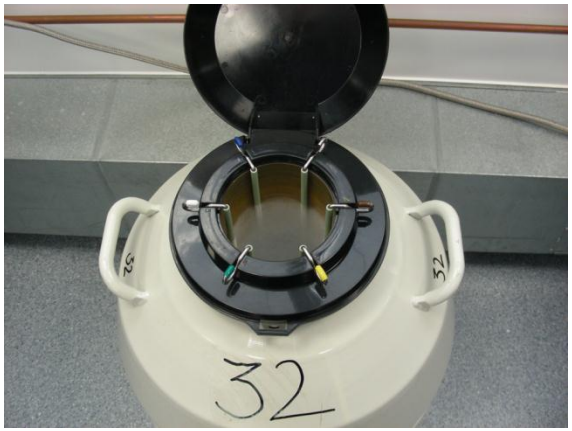


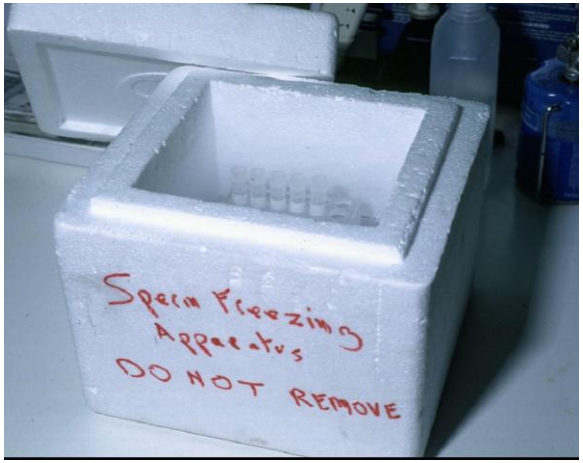
Mono-thioglycerol treated sperm

- 477 µM Mono-thioglycerol added to CPA
- 20µl aliquots of sperm and frozen in straws

Vial				Straw			
Groups	No. Tested	Fertilization Rate (%)	Range of FR (%)	Groups	No. Tested	Fertilization Rate (%)	Range of FR (%)
				129	3	24.1	5.9 - 44.3
C3H/HeH	20	29.5	7.1- 68.2	C3H/HeH	23	54.8	7.8 - 94.0
C57BL/6J	N/A	<5.0	<5.0	C57BL/6J	42	36.0	6.5 - 88.9

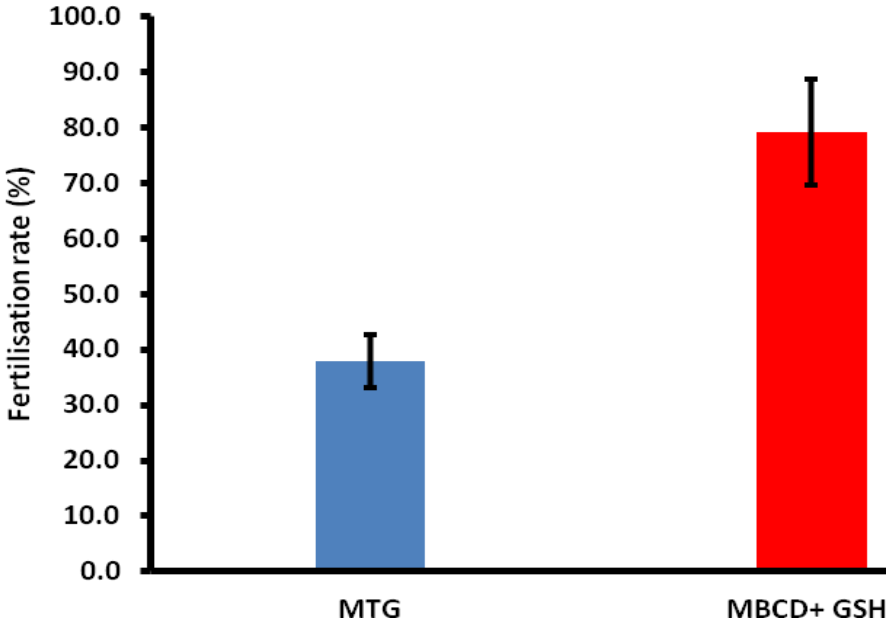
Sperm freezing equipment





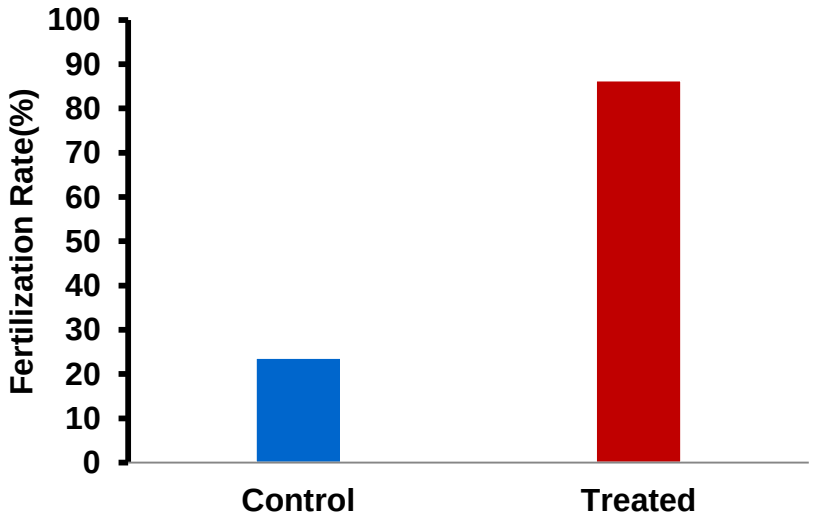
The addition of GSH to high Ca^{++} HTF (frozen sperm)

Group	Trial 1	Trial 2	Trial 3	Mean (%)
MTG	38.5	45.7	29.3	37.8
MBCD + GSH	82.8	93.2	61.0	79.0



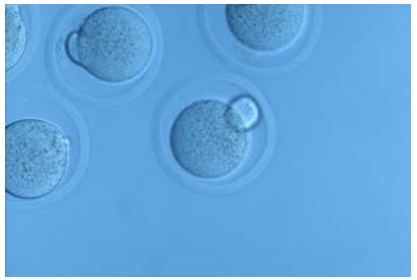
The addition of GSH to high Ca^{++} HTF (freshly harvested)

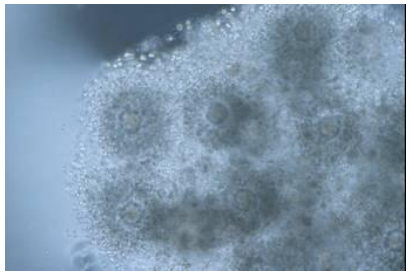
Group	No. of females used	No. of oocytes harvested	No. of embryos used in IVF	No. of 2-cells produced	Fertilisation rate (%)
Control	18	358	338	80	23.7
Treated	22	418	373	322	86.3



Handling poor sperm samples

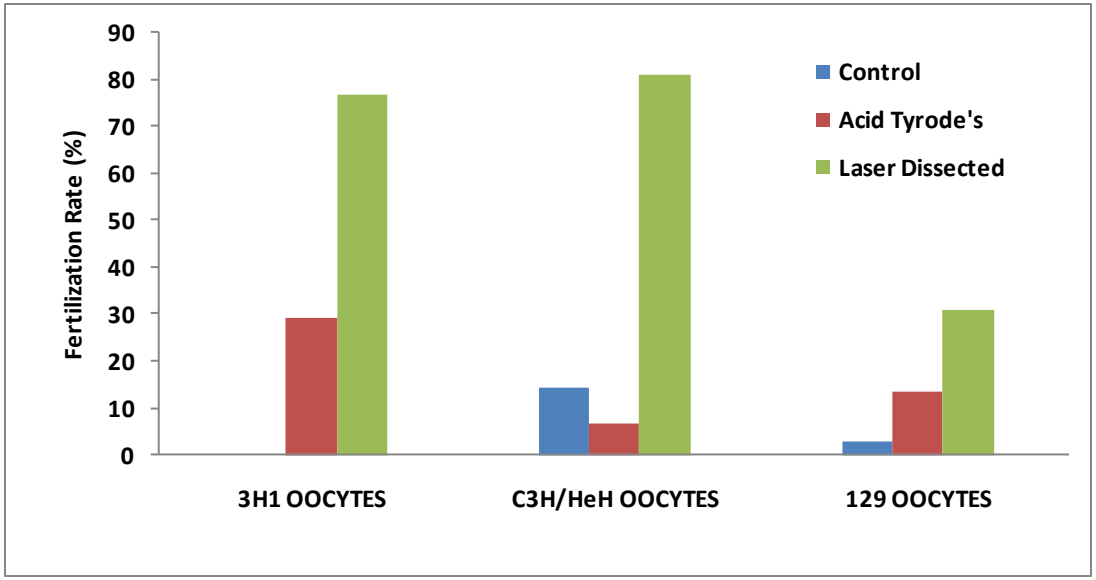
- Micro-insemination (Intra-cytoplasmic sperm injection)
- Laser assisted zona drilling
  - XYclone laser – Hamilton Thorne
- Partial zona dissection
  - Nakagata et al (1997) Biol. Reprod. **57**, 1050
- Zona thinning with acid tyrode's solution (pH 3.5)
  - Personal communication (A Doyle; TJL)
- Selection of motile sperm, plus removal of cell debris
  - Bath (2003) Biol. Reprod **68**, 19
- All methods require removal of the cumulus cells.



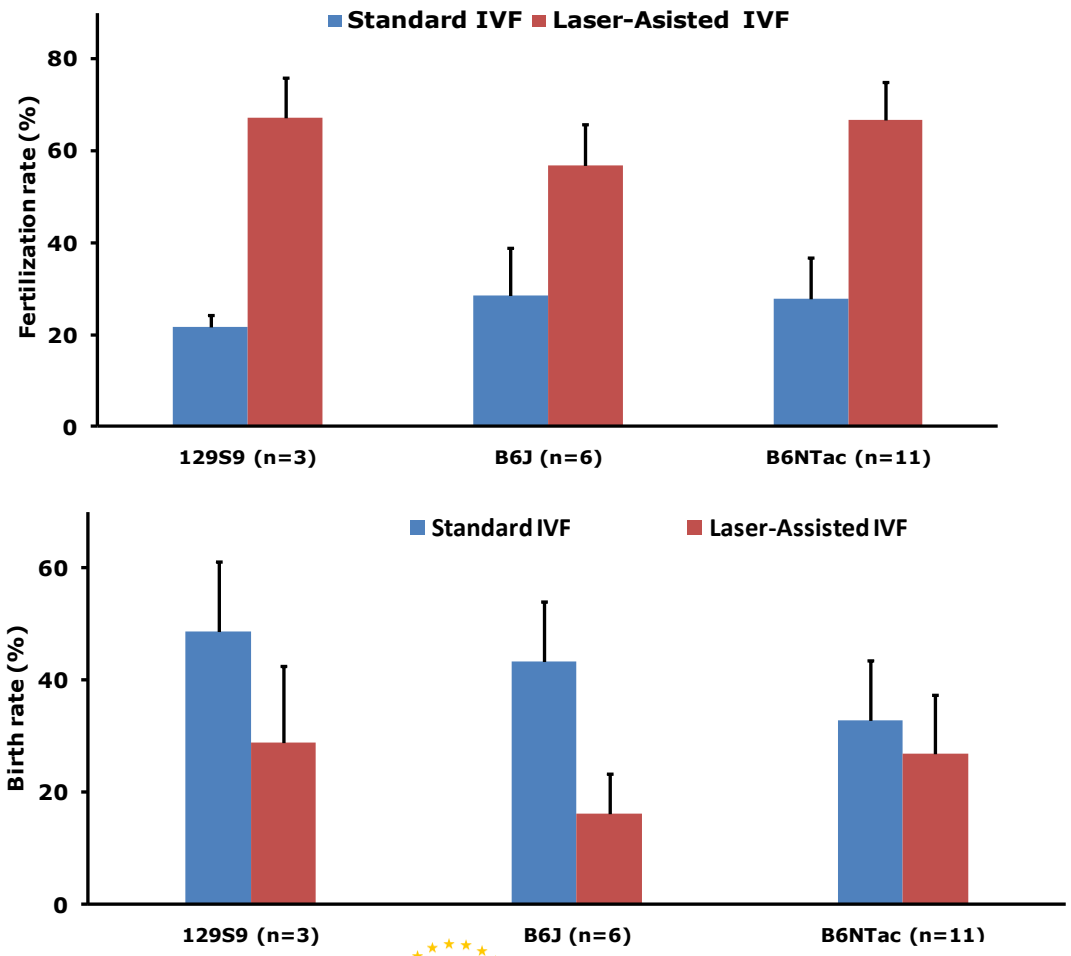


Laser versus normal IVF and Acid Tyrodes IVF

Groups	3H1 OOCYTES (n=1)			C3H/HeH OOCYTES (n=3)			129 OOCYTES (n=3)		
	No. Oocytes	2-cellS	FR%	No. Oocytes	2-cells	FR%	No. Oocytes	2-cellS	FR%
Control				99	14	14.1	68	2	2.9
Acid Tyrode's	114	33	28.9	90	6	6.7	775	103	13.3
Laser Dissected	39	30	76.9	99	80	80.8	369	114	30.9

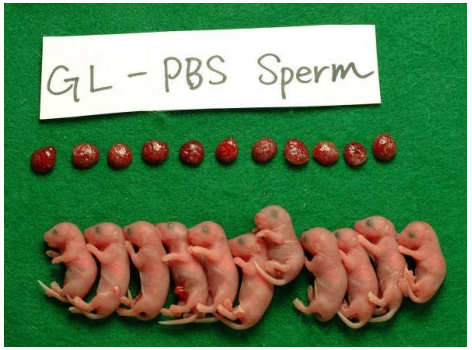


Laser treatment across different backgrounds



Freezing without cryoprotectants

- Freeze dried sperm stored at 4ºC
  - Ward et al (2003) Biol. Reprod. **69**, 2100
- Spermatozoa/spermatids retrieved from reproductive tissues
  - Ogonuki et al (2006) PNAS, **103**, 13098
- Freezing in EDTA /Tris-HCL buffered saline
  - Ward et al (2003) Biol. Reprod. **69**, 2100
- Micro-insemination is required to recover live mice ICSI



Bio-security

- Most microbial (viral, bacterial & protozoal) agents are removed by ET
- Special cases:
  - Mycoplasma
  - LCMV
  - Parvovirus?
- Wash embryos before transplantation – IETS recommendations

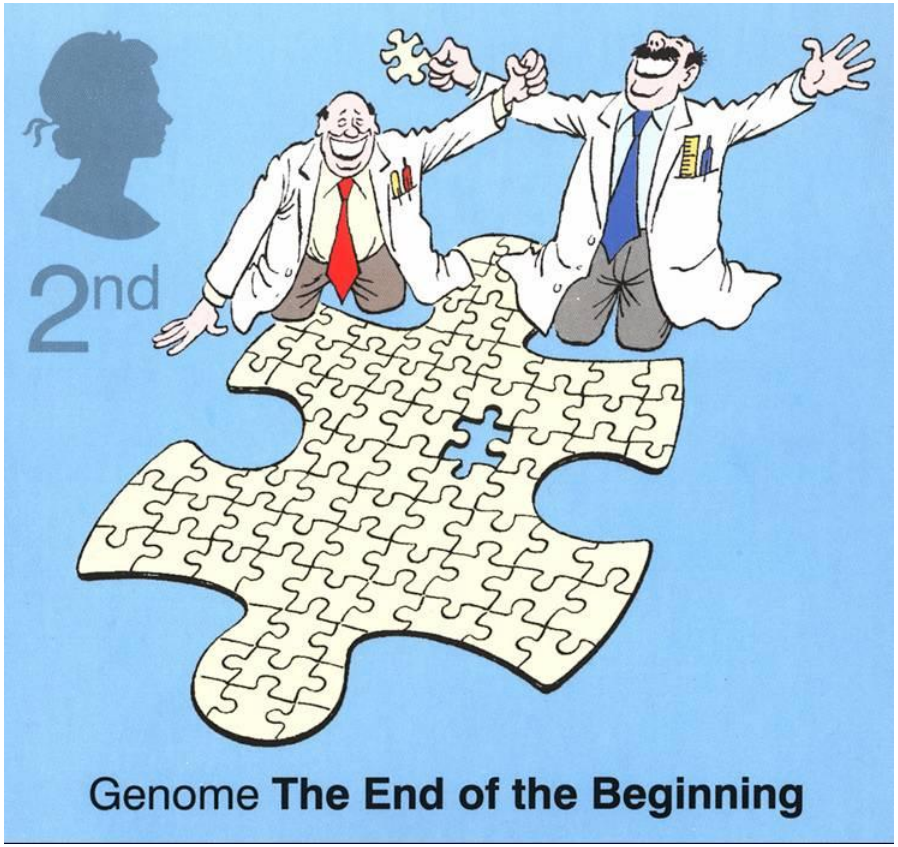
Archiving Summary:

Embryos

- Well proven, >35 years
- Success not particularly strain dependent
- Requires large numbers of mice
- Requires skilled personnel
- Dissemination is relatively easy

Sperm

- New technology, ~15 years
- Success dependent on genetic background
- Only haploid genotype, requires oocytes for IVF
- Simple, rapid & cheap
- IVF more skilful
- Dissemination more difficult


2nd
Genome The End of the Beginning