

# Harwell Embryo and Spermatozoa Cryopreservation Training Course

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Medical Research Council  
Harwell, UK**



An International Centre for Mouse Genetics



# The MRC frozen embryo archive

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- Worldwide Genetic Resource
  - ~1450 stocks, >470,000 embryos
    - Includes transgenic, mutants, chromosome anomalies & inbred strains
- 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)**

# Mary Lyon Centre – high barrier unit

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# Course aims

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- Hands on demonstration of:
  - Embryo freezing
  - Sperm freezing
  - *In vitro* fertilization
  
- Reference point
- Disseminate skills



# Handling liquid nitrogen

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

# What can be cryopreserved?

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



# Benefits of cryopreservation

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- 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



# Store stocks in duplicate

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# Data management

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

# Landmarks in cryopreservation: 1

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



# Landmarks in cryopreservation: 2

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- 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  $-79^{\circ}\text{C}$
  - Obtained live mice after orthotopic transplantation of the thawed tissue
  - **First incidence of live mice from cryopreserved materials**



# Landmarks in cryopreservation: 3

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- 1971: David Whittingham
  - Reported live mice born from embryos frozen to  $-79^{\circ}\text{C}$  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  $\text{LN}_2$
- 1972: Whittingham, Leibo & Mazur
  - **Many live mice from embryos frozen in 1M DMSO in  $\text{LN}_2$**



# Landmarks in cryopreservation: 4

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- 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

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- Embryos stored under low-dose  $\gamma$  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
  - Simulated storage of up to 2000 yr. under normal levels of background radiation

# Recovery of genetic variants

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- 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 ( $aa\ bpbp\ fz fz\ InIn\ papa\ pepe$ )
  - XO (tagged with  $Ta$  &  $Mo^{blo}$ )

# Brief history of mouse sperm cryopreservation

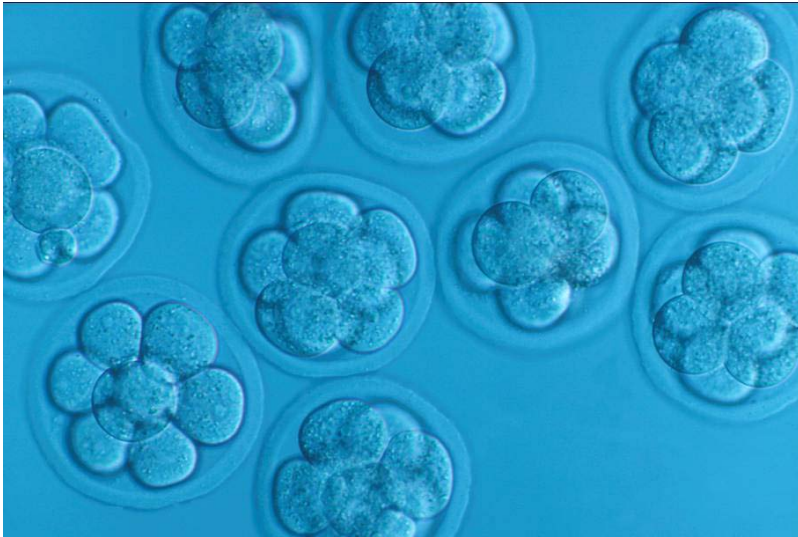
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- 1990: Yokoyama *et al* used various combinations of raffinose, glycerol, DMSO and skim milk
- 1990: Tada *et al* used 18% raffinose in saline. Also glycerol and DMSO
- 1990: Okuyama *et al* used 18% raffinose plus 3% skim milk
- 1992: Nakagata & Takeshima modified cryoprotectant elimination
- **2008: Ostermeier et al used monothioglycerol to improve recovery rates from B6 & 129 sperm**



# Embryo freezing at Harwell

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# Cryopreservation of the pre-implantation embryo

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- Controlled rate freezing
- Vitrification



# Key aspects of cryopreservation

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



# Types of cryoprotectants

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- **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**

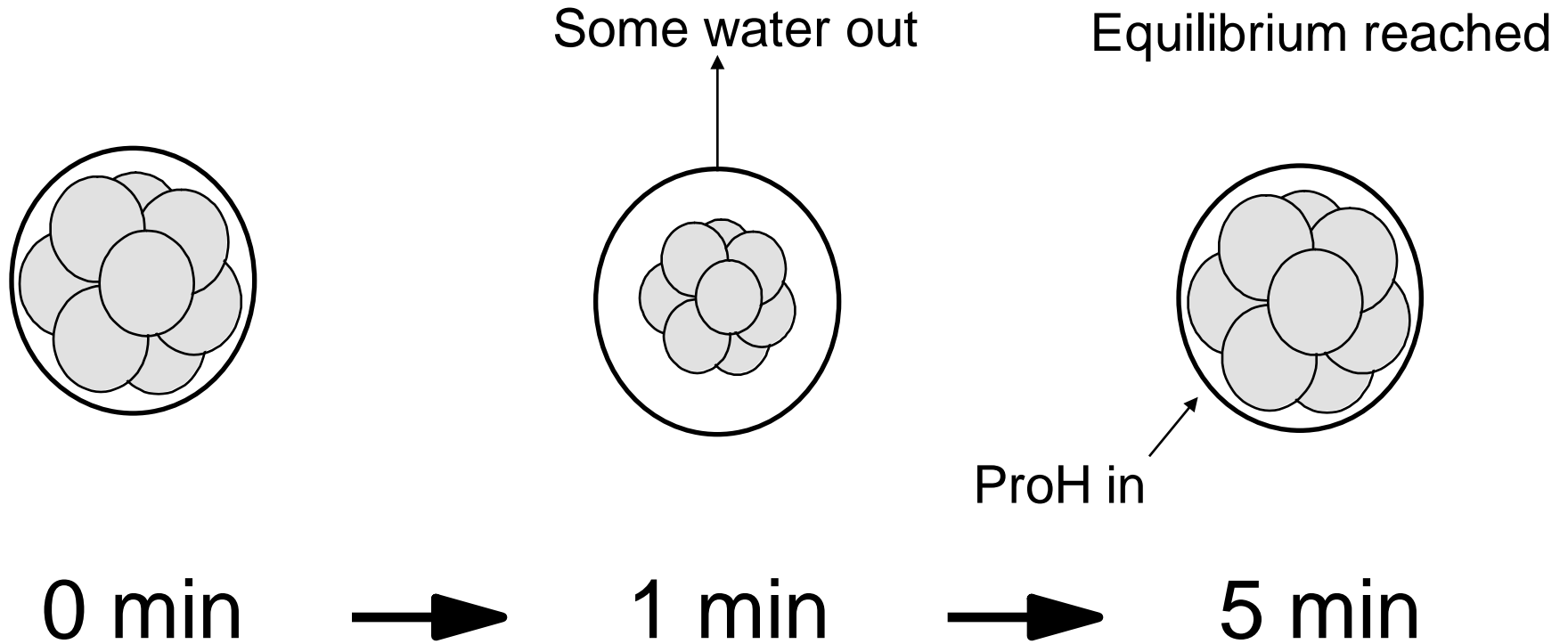


# Embryo cryopreservation: protocol

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- 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

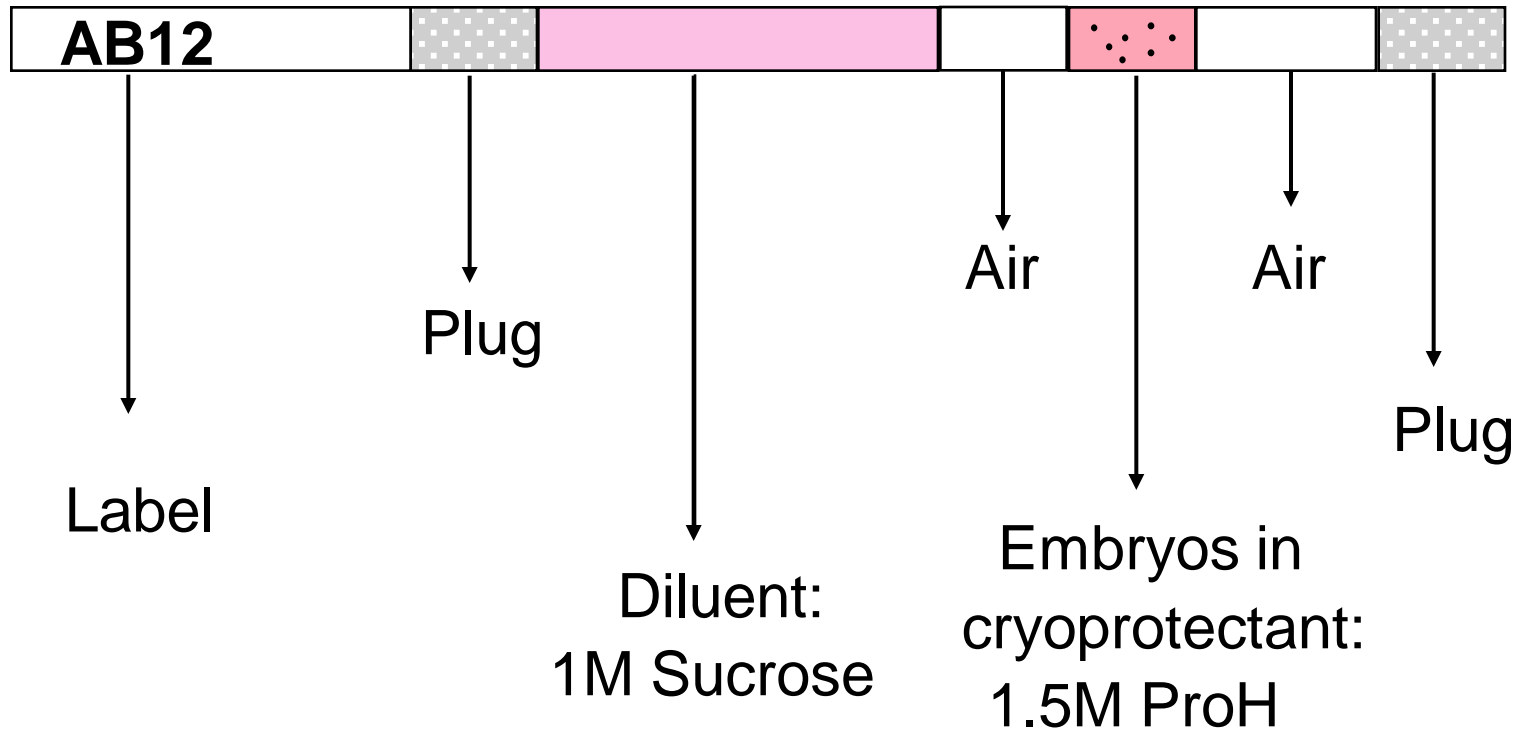
# 8-cell embryo in 1.5M ProH



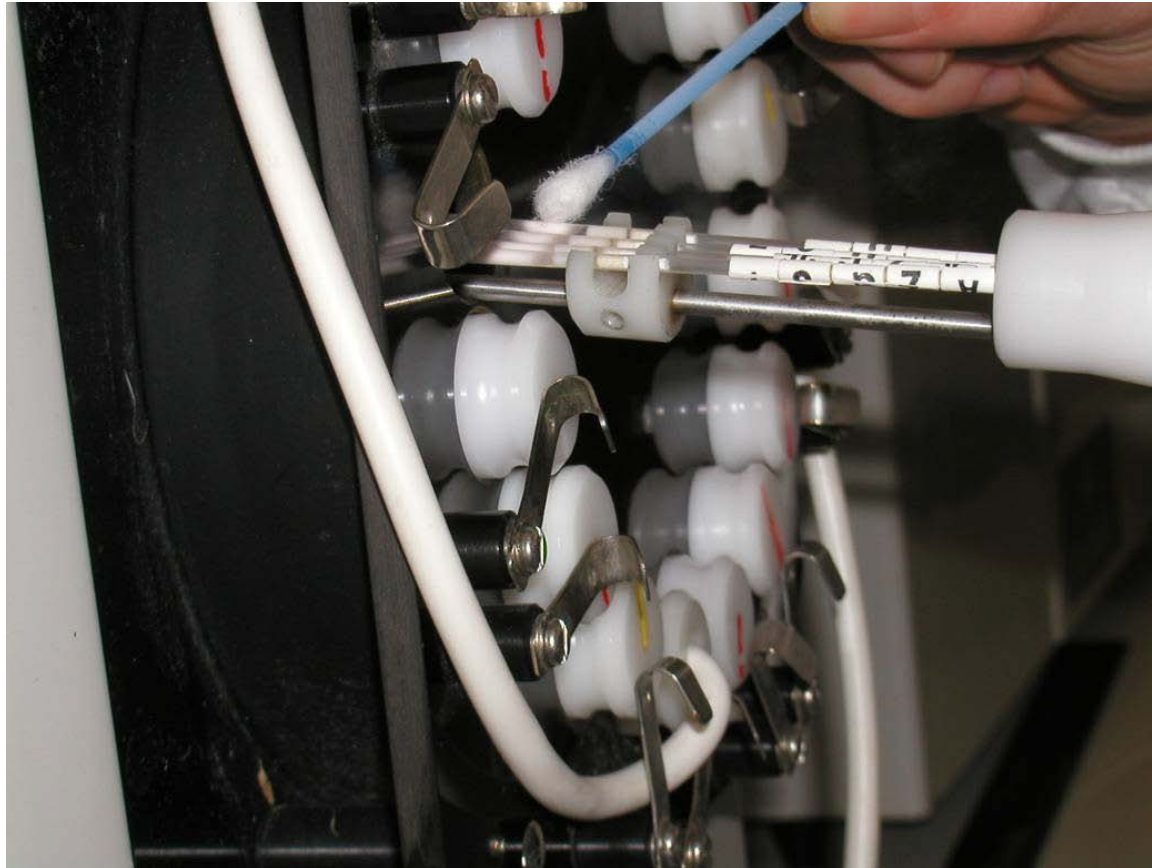
# Loading embryos



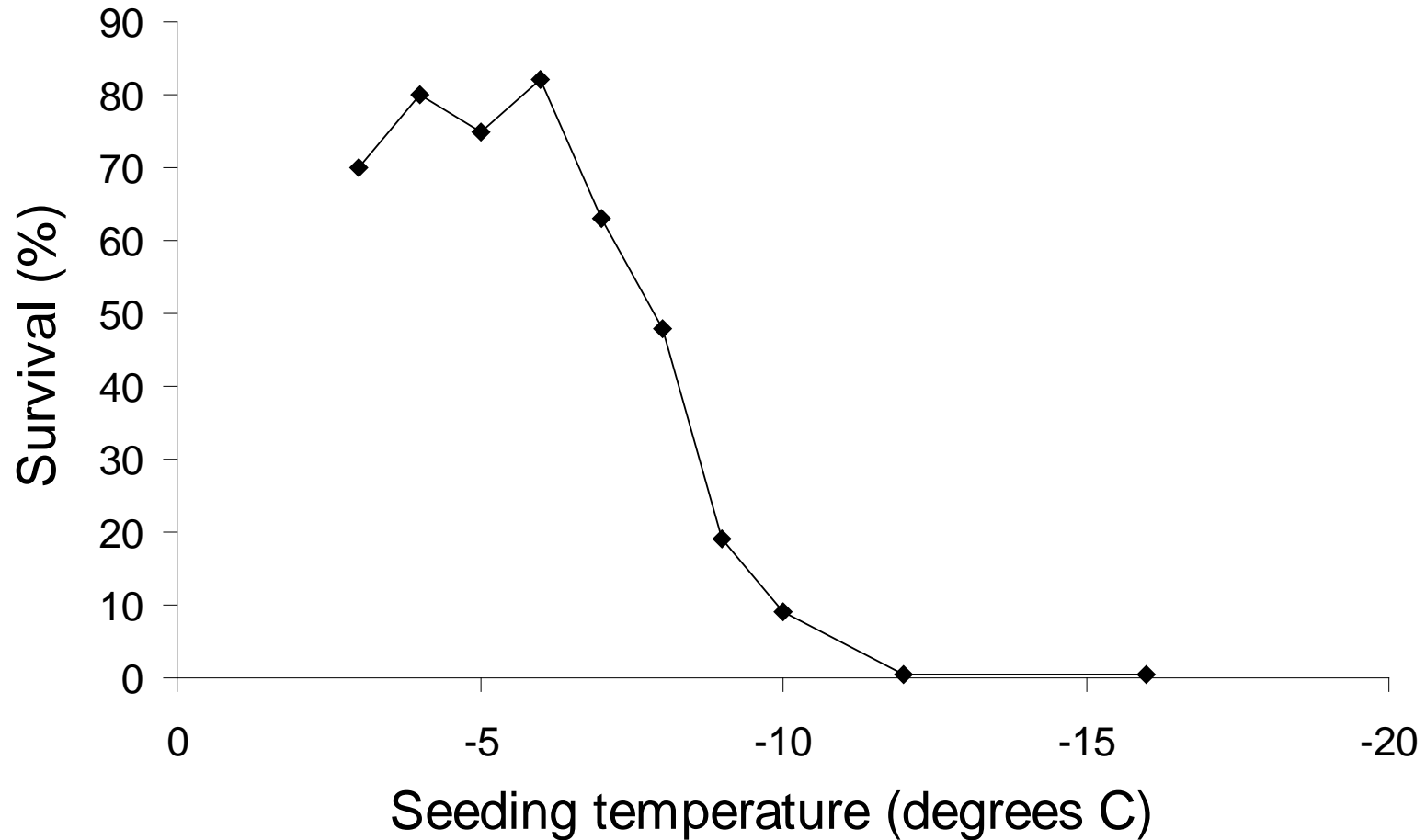
# Embryos frozen in plastic semen straws



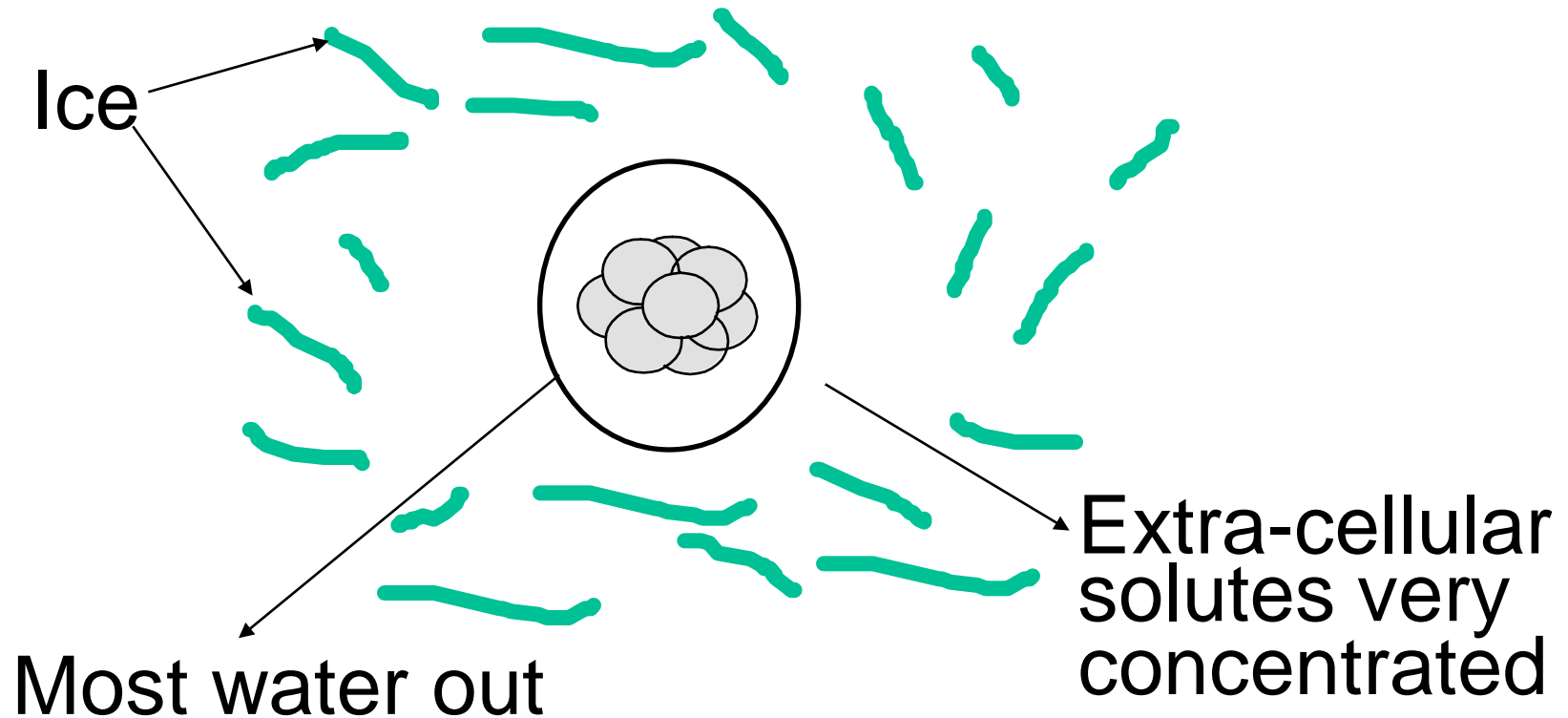
# Seeding the straws



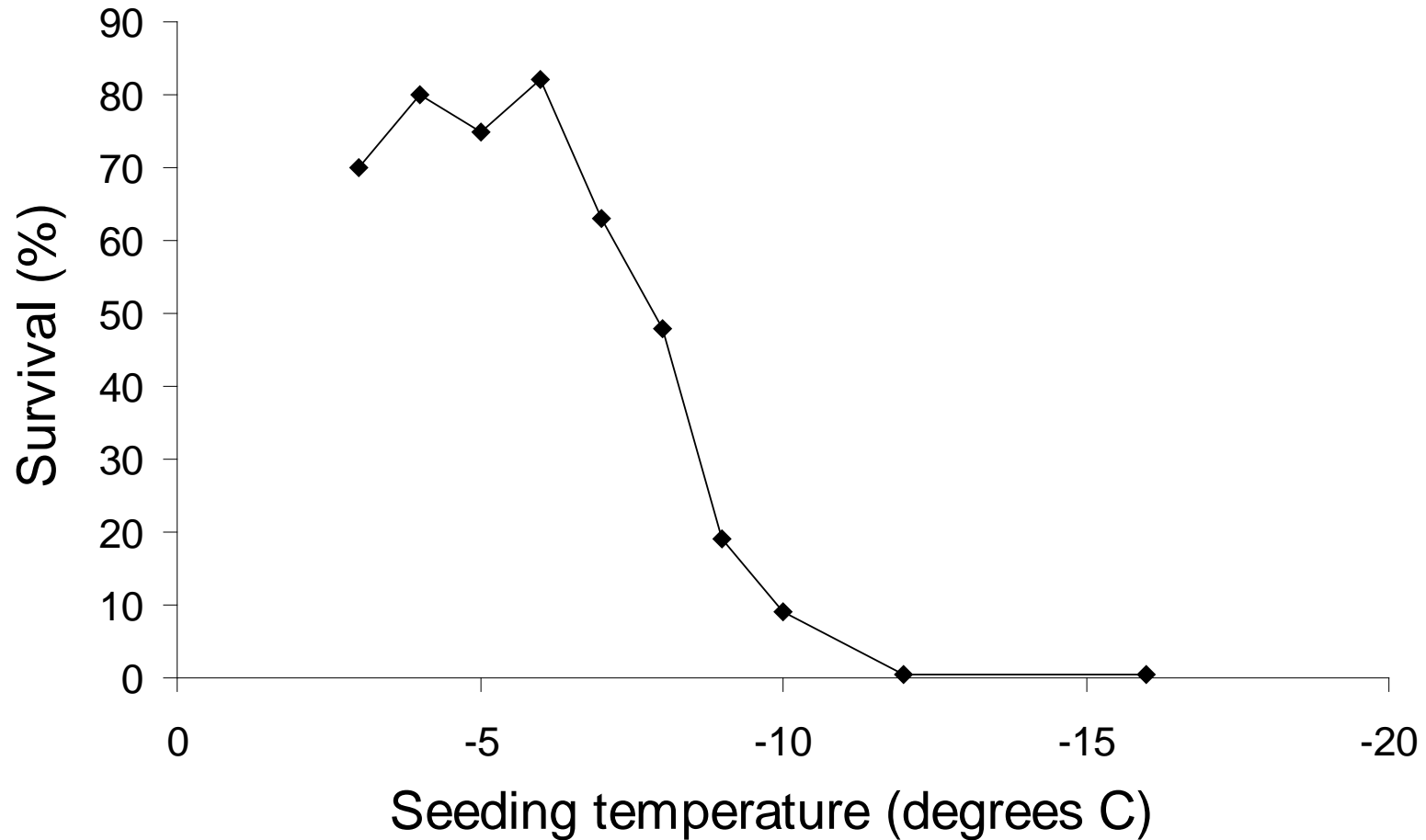
# Effect of seeding temperature



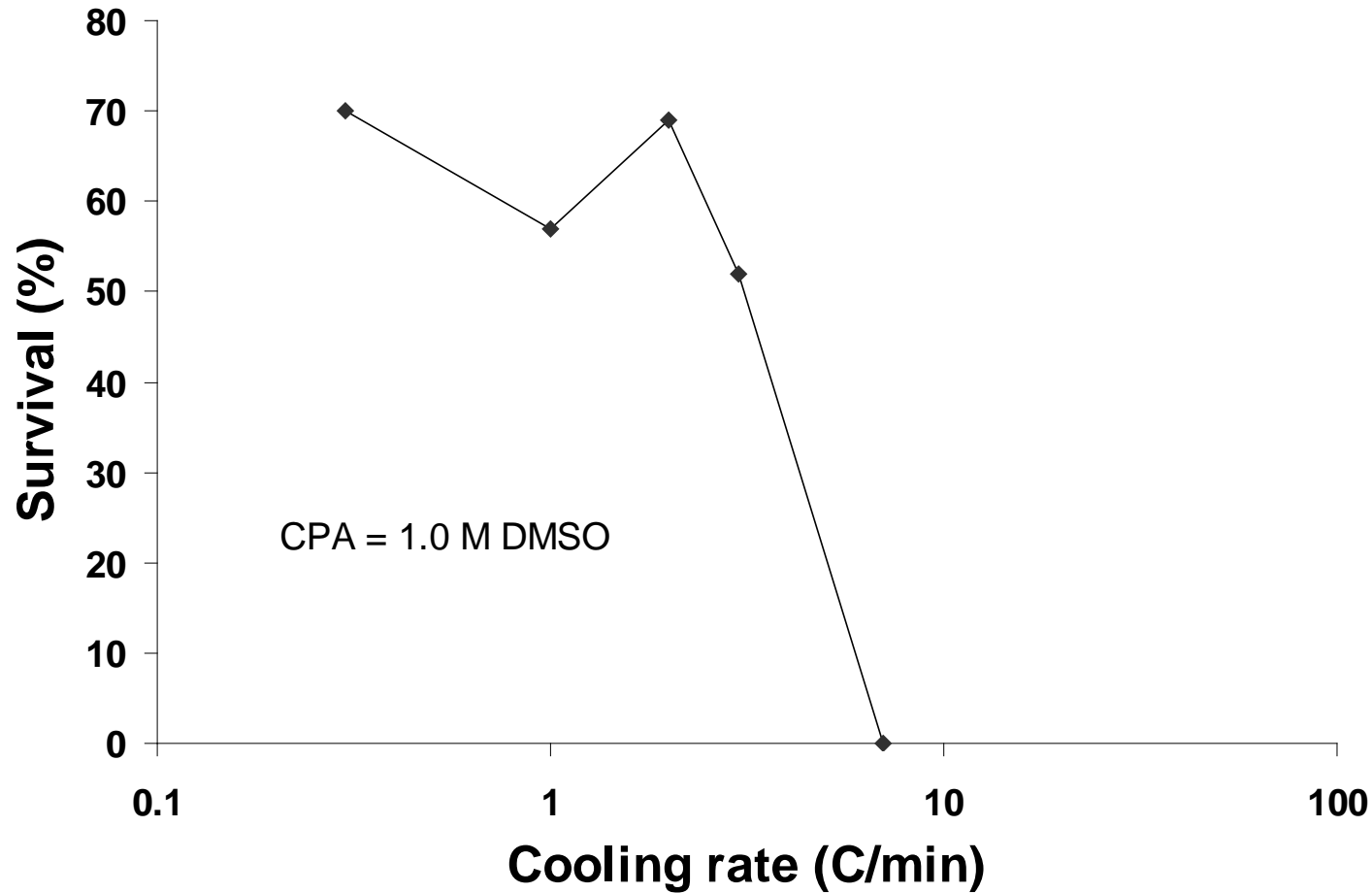
# 8-cell embryos cooled to $-30^{\circ}\text{C}$ at $0.3^{\circ}\text{C}/\text{min}$ .



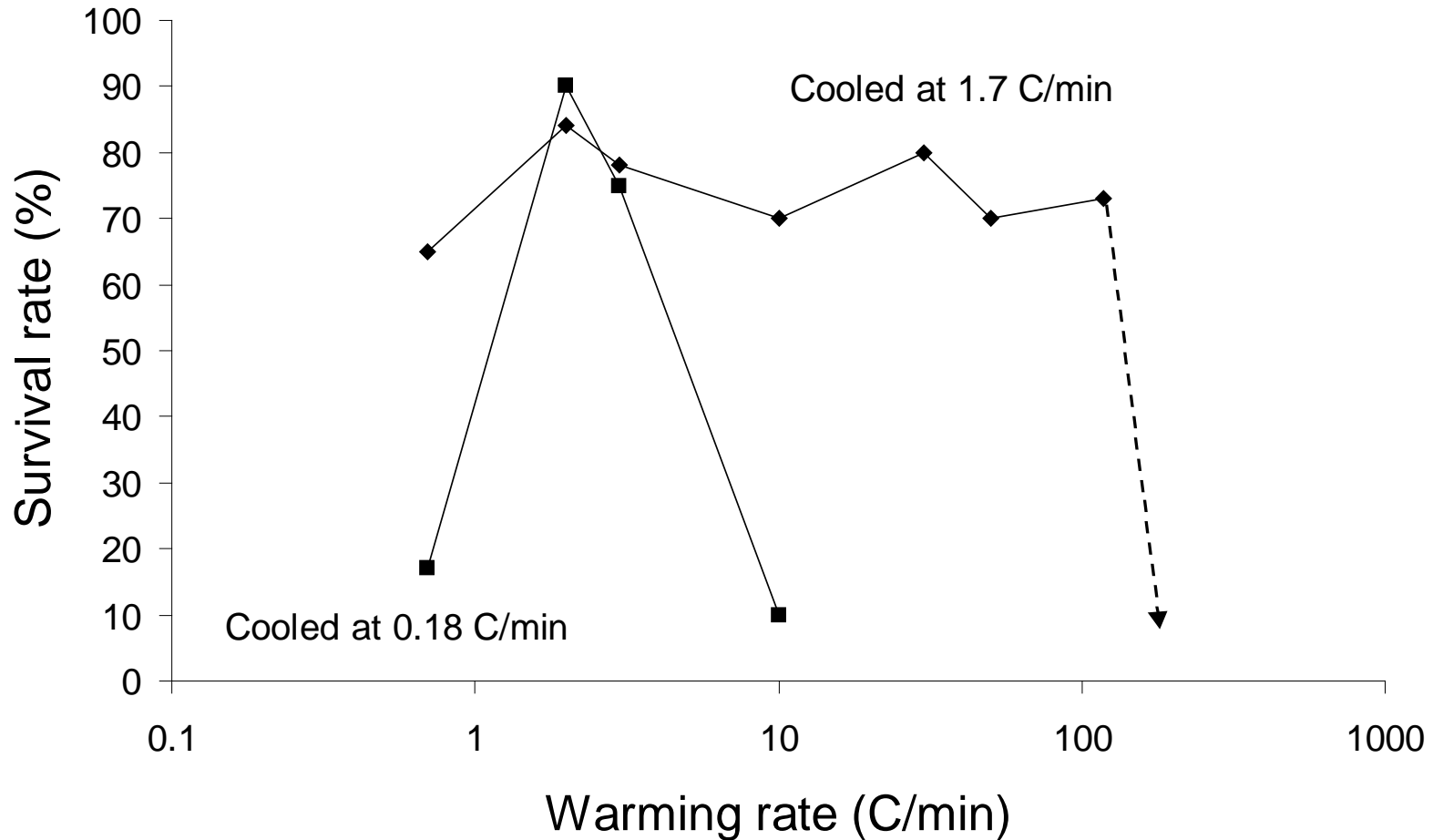
# Effect of seeding temperature



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

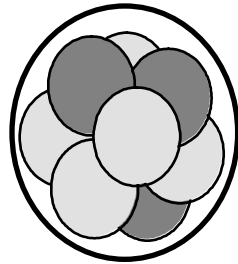


# Effect of warming rate - Whittingham *et al* (1972)



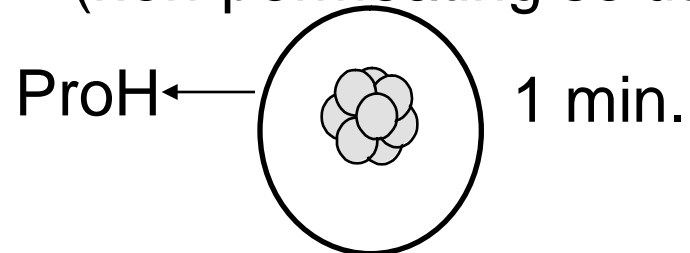
# Embryo shortly after rapid warming from $-196^{\circ}\text{C}$

**No Sucrose**



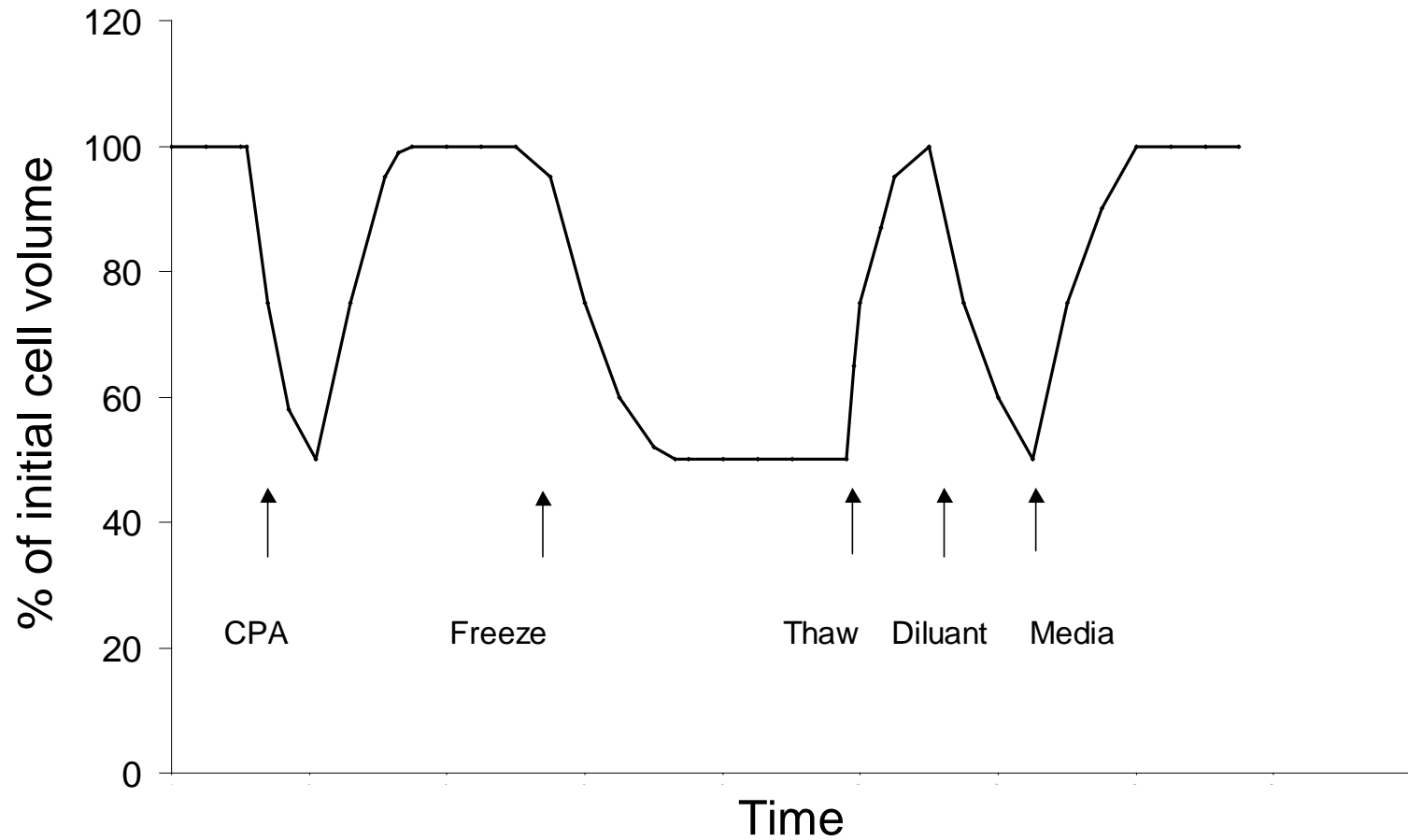
Rapidly swollen embryo containing ProH and water (damaged)

**1.0M Sucrose**  
(non-permeating solute)



Isotonic solution.  
5 min.

# Embryo freezing dynamics



# Cryopreservation of mouse sperm

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- Low tech in comparison with embryo freezing

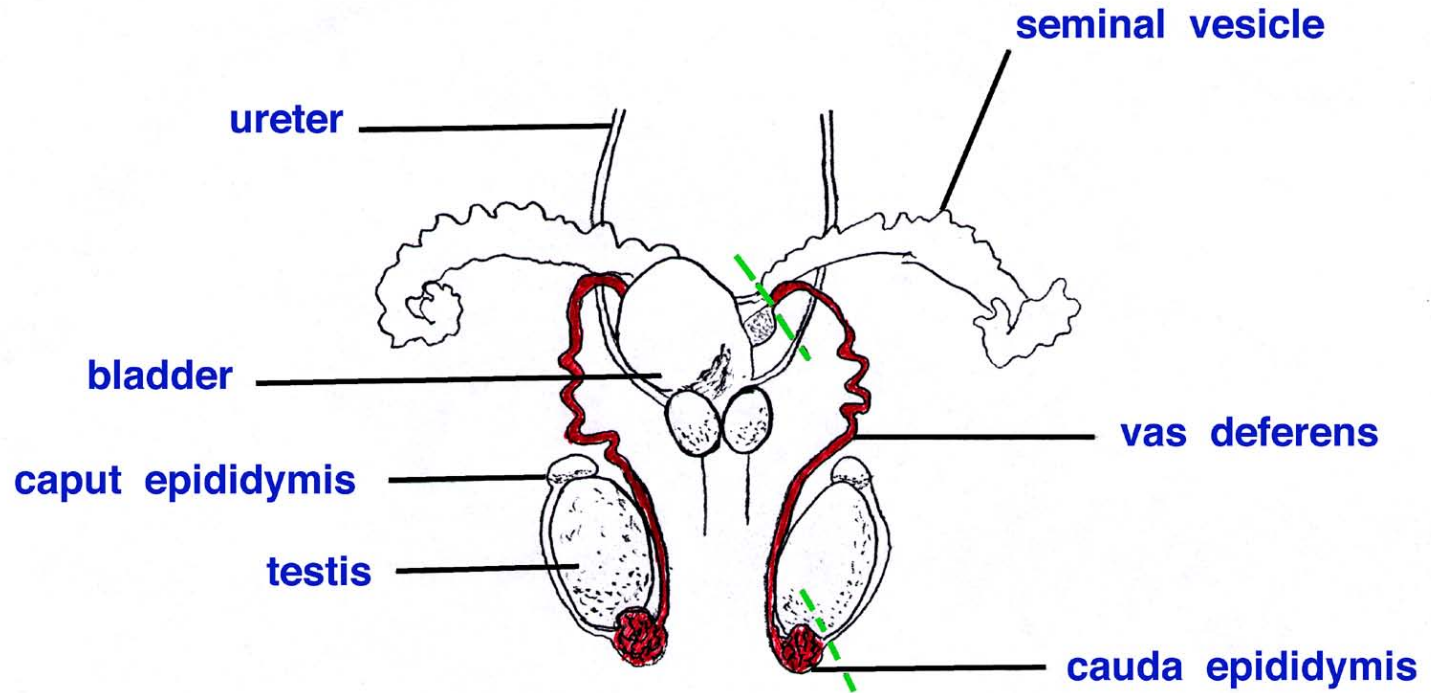


# Sperm freezing: applications

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- Archiving, plus DNA library
- Export/Import mutants
- Cheap and easy
- Rapidly freeze down stock

# Urinogenital system of mouse



# Sperm cryopreservation method

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- Mince cauda and vas in 1ml 18% raffinose, 3% skim milk
- Incubate at 37°C for 10 min
- 100µl aliquot placed in cryotubes
- Place tubes in LN<sub>2</sub> vapour for 10 min.
- Plunge into LN<sub>2</sub> and store until required
  
- Thaw sperm in air for 30sec, then plunge into 37°C water bath

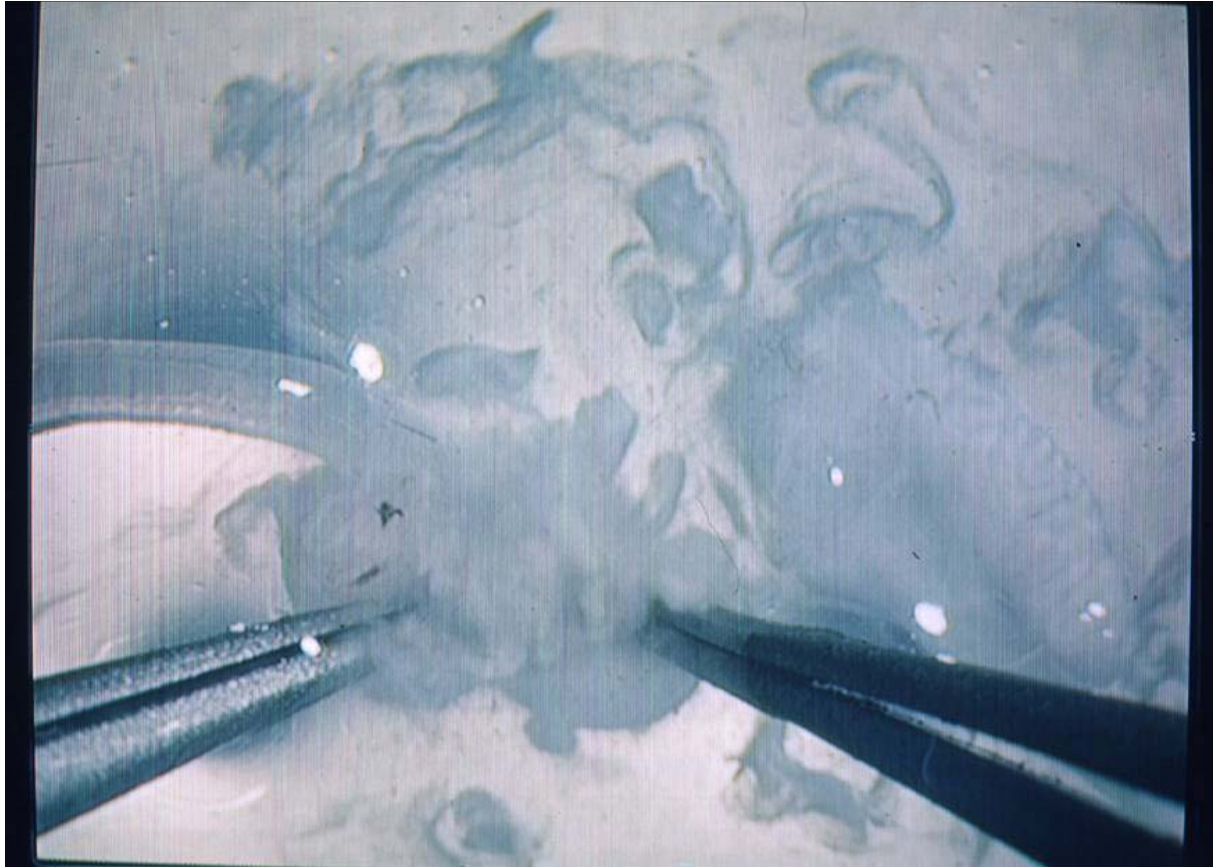
# Dissected cauda & vas

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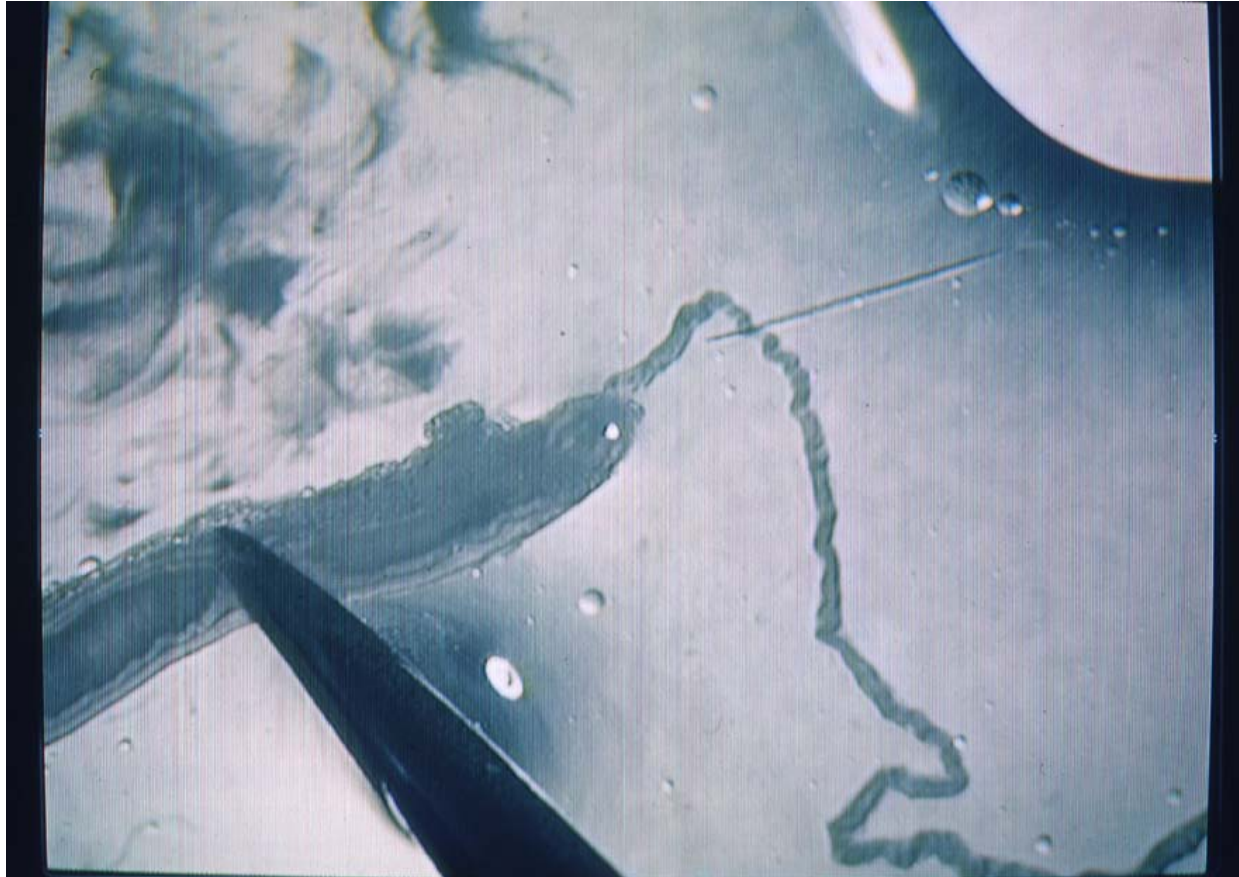
# Releasing sperm from cauda

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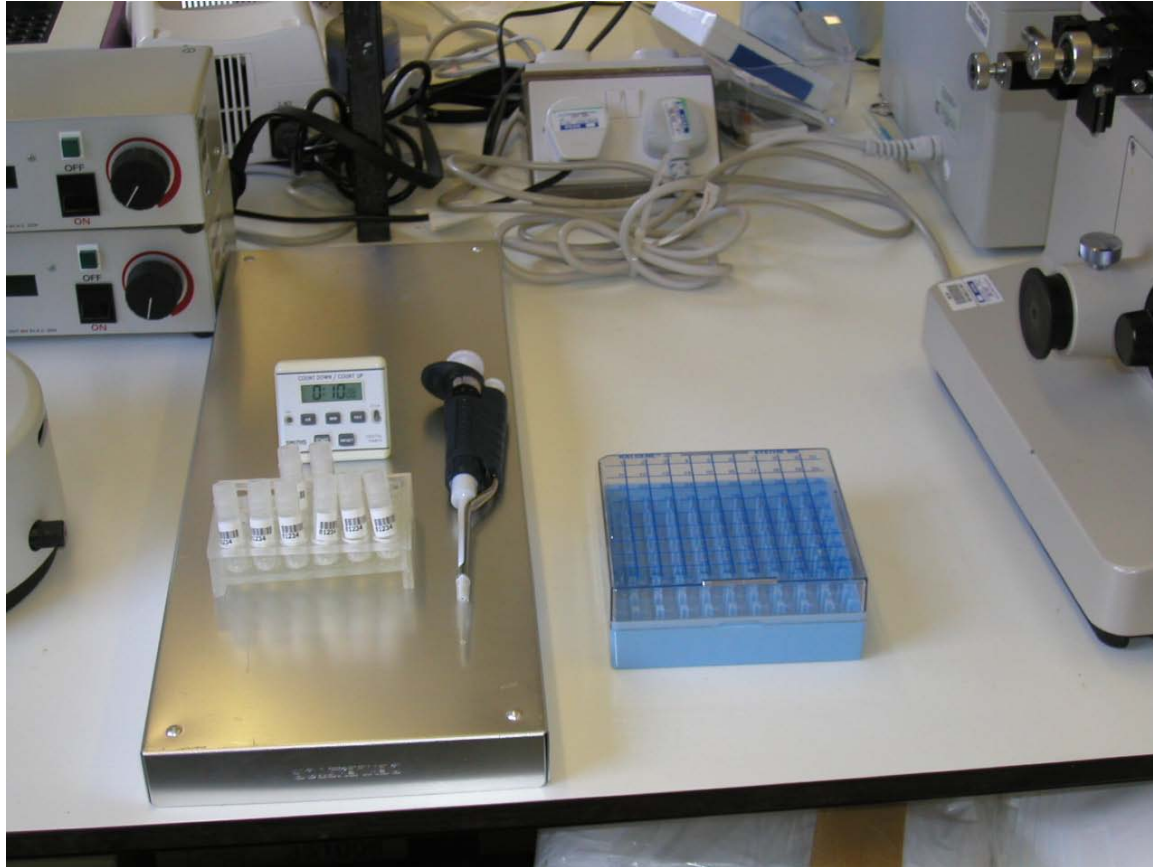


# Releasing sperm from vas

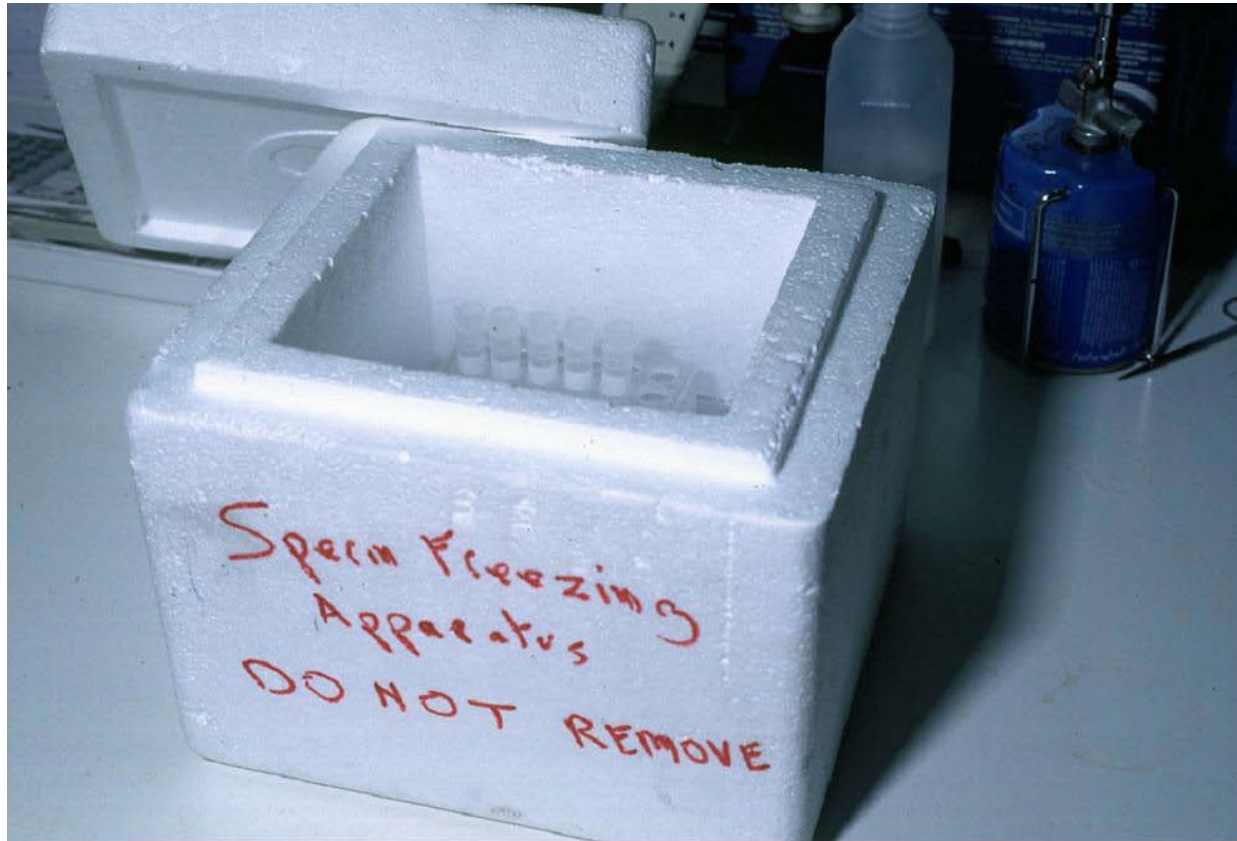
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# Sperm freezing equipment

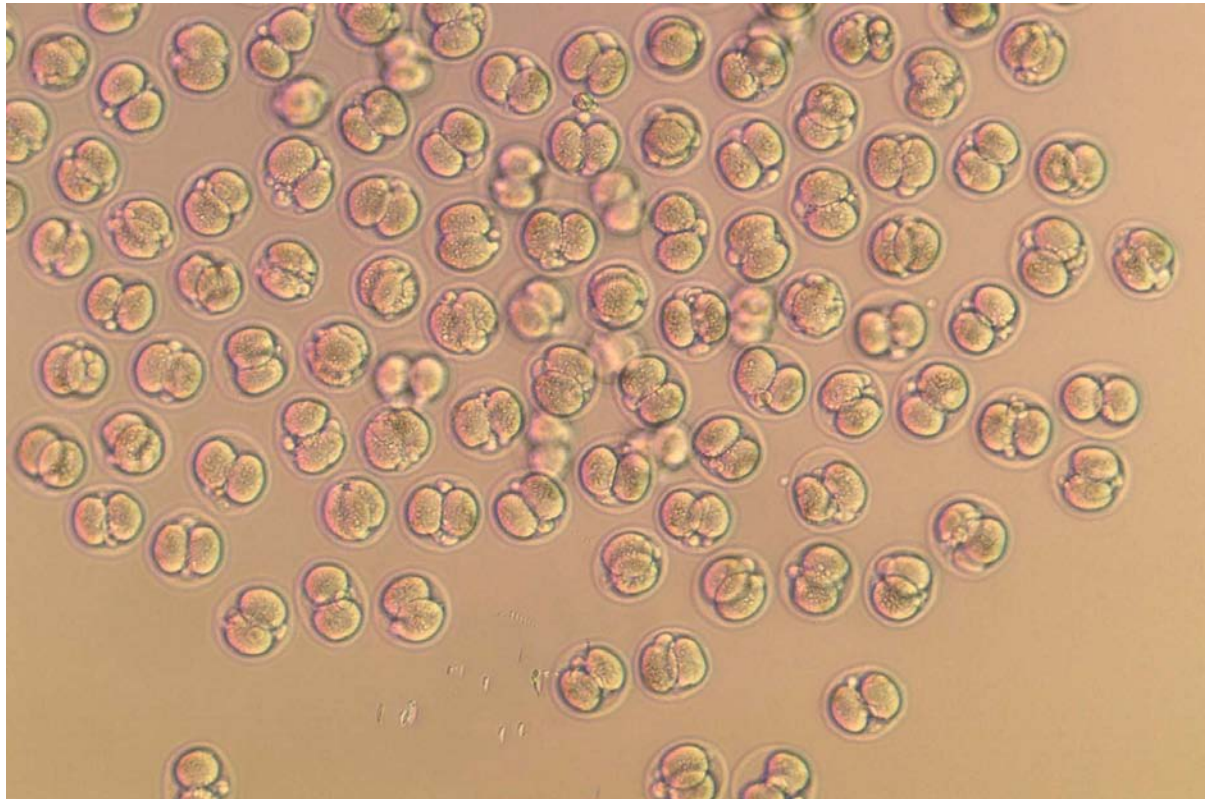


# Sperm freezing apparatus



# *In vitro* fertilization

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# Exploitation of *in vitro* fertilization

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- 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

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- Warm frozen tube rapidly in 37°C
- Pipette aliquots (5-10 $\mu$ l) of sperm into pre-incubated 500 $\mu$ l drops Cooks
- Add up to 6 cumulus masses; ~14 hours post hCG
- Incubate ~5 hours, 37°C, 5% CO<sub>2</sub> in air
- Wash eggs, culture overnight in 150 $\mu$ l Cooks
- Transfer 2-cell embryos to oviducts of 0.5 day pseudopregnant recipients

# Potential of IVF using frozen sperm

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- 1 x 10 $\mu$ l aliquot of frozen (C3H/HeH x BALB/c)F1 sperm used to fertilise 420 (C3H/HeH x 101/H)F1 oocytes *in vitro*
- 239, 2-cell embryos obtained (57%)
- 112 transferred to 7 recipient females, 67 animals born (60% of transferred)
- If all frozen sperm used in similar IVF's, we predict ~6,700 offspring from this male

# Sperm cryopreservation: extrapolation

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- Theoretically possible to recover 5,000-10,000 mice from the frozen sperm of one male
- Limiting Factors:
  - No. of eggs available for IVF
  - No. of recipient females
  - Genotype dependent

# Frozen BALB/c sperm

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- Male 1:
  - 811 (C3H/HeH x 101/H)F1 eggs
  - 30; 2-cell embryos transferred
  - 4 liveborn offspring
- Male 2:
  - 290 (C3H/HeH x 101/H)F1 eggs
  - 5; 2-cell embryos transferred
  - 0 liveborn offspring

# Frozen C57BL/6J sperm

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- 1,132 (C3H/HeH x 101/H)F1 eggs
- 116; 2-cell embryos transferred
- 0 liveborn offspring

# Freezing without cryoprotectants

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- Freeze dried sperm stored at 4°C
  - 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



# Handling poor sperm samples

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- Micro-insemination (Intra-cytoplasmic sperm injection)
- Partial zona-pellucida dissection
  - Nakagata et al (1997) Biol. Reprod. **57**, 1050
- Zona thinning with acid tyrodes 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.

# Bio-security

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

# Archiving Summary:

## Embryos

## Sperm

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



