17th Harwell Embryo and Spermatozoa Cryopreservation Training Course


Martin Fray

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](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 Centre for Mouse Genetics
An international service

Total Requested Material

- Frozen (469) 25.91%
- Live (1,320) 73.09%

Requests per Country

Country: United States, Germany, France, United Kingdom, Italy, China, Japan, Canada, Spain, Switzerland, Netherlands, Belgium, Australia, Sweden, Ireland, Israel, Czech Republic, Greece, Denmark, Portugal, Estonia, South Korea, Poland, Hungary, Taiwan, Cyprus, New Zealand, Norway, Poland, Brazil, Croatia, Slovenia

Requests: 706, 293, 263, 201, 92, 71, 53, 41, 38, 33, 29, 28, 27, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, 0
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

![Graphs showing temperature changes over days for both vertical and horizontal orientations of Dry Shipper](image-url)
Mouse information sheets

An International Centre for Mouse Genetics

MRC

EMMA

100 years of life-changing discoveries
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°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 $-79^\circ$ 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 °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 LN₂

- 1972: Whittingham, Leibo & Mazur
  - Many live mice from embryos frozen in 1M DMSO in LN₂
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 $\gamma$ 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
  - 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

0 min → 1 min → 5 min

Some water out
Equilibrium reached

ProH in
Loading embryos
Embryos frozen in plastic semen straws

- AB12
- Plug
- Label
- Diluent: 1M Sucrose
- Air
- Embryos in cryoprotectant: 1.5M ProH
- Air
- Plug
Seeding the straws
Effect of seeding temperature

Seeding temperature (degrees C) vs. Survival (%)

- Survival decreases significantly as the seeding temperature decreases.
- The highest survival is observed at temperatures around 0°C.
- Survival drops to nearly 0% at temperatures below -10°C.
8-cell embryos cooled to -30°C at 0.3 to 0.5°C/min.

- Ice
- Most water out
- Extra-cellular solutes very concentrated
Effect of seeding temperature

Seeding temperature (degrees C)

Survival (%)

Survival (%)

Seeding temperature (degrees C)
Effect of cooling rate - Whittingham et al (1972)

CPA = 1.0 M DMSO
Effect of warming rate - Whittingham et al (1972)

![Graph showing the effect of warming rate on survival rate. The graph compares survival rates for samples cooled at 0.18°C/min and 1.7°C/min. The survival rate decreases significantly with higher warming rates.]
Embryo shortly after rapid warming from -196°C

No Sucrose

Rapidly swollen embryo containing ProH and water (damaged)

1.0M Sucrose (non-permeating solute)

ProH → 1 min.

Isotonic solution.

5 min.
Embryo freezing dynamics

% of initial cell volume

CPA  Freeze  Thaw  Diluant  Media

Time

0  20  40  60  80  100  120

Time
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

<table>
<thead>
<tr>
<th>Order</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artiodactyla</td>
<td>24 species: bovid, camel, deer</td>
</tr>
<tr>
<td>Carnivora</td>
<td>14 species: cat, dog, cheetah, fox</td>
</tr>
<tr>
<td>Cetacea</td>
<td>1 species: dolphin</td>
</tr>
<tr>
<td>Lagomorpha</td>
<td>1 species: rabbit</td>
</tr>
<tr>
<td>Perissodactyla</td>
<td>1 species: horse</td>
</tr>
<tr>
<td>Rodentia</td>
<td>2 species: mouse, rat</td>
</tr>
<tr>
<td>Primates</td>
<td>7 species: human, gorilla, chimp</td>
</tr>
</tbody>
</table>
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

- **ureter**
- **bladder**
- **caput epididymis**
- **testis**
- **vas deferens**
- **cauda epididymis**
- **seminal vesicle**
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 LN₂
- Plunge in LN₂

- Thaw sperm in by plunging into 37°C water bath for 10 mins
Sperm freeze method - 2

Collection of cauda epididymides

Preparation of sperm suspension
Cauda epididymides
120 µl CPA
Paraffin oil

Aspirate HTF solution
HTF

Load the straw
heat sealed
aliquots of sperm
10ul
HTF
cotton plug

100 years of life-changing discoveries
Sperm freeze method - 3

10 minutes
Sperm freezing profile

![Graph showing cooling rate vs time for different depths.]

### Groups

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

**LN₂**
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 37°C for 10 mins
- Disperse 10μl of sperm into a 90μl drop of TYH + 0.75mM MBCD for 30mins at 37°C
- Add up to 6 x cumulus masses; ~14 hours post hCG
- Use high Ca²⁺ HTF supplemented with 0.25mM GSH
- Incubate for 3 to 5hrs, 37°C, 5% CO₂ in air
- Wash eggs, culture overnight in 150μl high Ca²⁺ HTF
- Transfer 2-cell embryos to oviducts of 0.5 day pseudopregnant recipients
Potential of IVF using frozen sperm – (MBCD method)

- 10 x 10 μl 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

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. Tested</th>
<th>Fertilization Rate (%)</th>
<th>Range of FR (%)</th>
<th>Groups</th>
<th>No. Tested</th>
<th>Fertilization Rate (%)</th>
<th>Range of FR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeH</td>
<td>20</td>
<td>29.5</td>
<td>7.1 - 68.2</td>
<td>C3H/HeH</td>
<td>23</td>
<td>54.8</td>
<td>7.8 - 94.0</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>N/A</td>
<td>&lt;5.0</td>
<td>&lt;5.0</td>
<td>C57BL/6J</td>
<td>42</td>
<td>36.0</td>
<td>6.5 - 88.9</td>
</tr>
</tbody>
</table>
Sperm freezing equipment
The addition of GSH to high Ca\textsuperscript{++} HTF (frozen sperm)

<table>
<thead>
<tr>
<th>Group</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
<th>Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTG</td>
<td>38.5</td>
<td>45.7</td>
<td>29.3</td>
<td>37.8</td>
</tr>
<tr>
<td>MBCD + GSH</td>
<td>82.8</td>
<td>93.2</td>
<td>61.0</td>
<td>79.0</td>
</tr>
</tbody>
</table>
The addition of GSH to high Ca\textsuperscript{++} HTF (freshly harvested)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of females used</th>
<th>No. of oocytes harvested</th>
<th>No. of embryos used in IVF</th>
<th>No. of 2-cells produced</th>
<th>Fertilisation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18</td>
<td>358</td>
<td>338</td>
<td>80</td>
<td>23.7</td>
</tr>
<tr>
<td>Treated</td>
<td>22</td>
<td>418</td>
<td>373</td>
<td>322</td>
<td>86.3</td>
</tr>
</tbody>
</table>

![Fertilization Rate Chart]

An International Centre for Mouse Genetics

EMMA

MRC Medical Research Council

100 years of life-changing discoveries
Handling poor sperm samples

- Micro-insemination (Intra-cytoplasmic sperm injection)

- Laser assisted zona drilling
  - XYclone laser – Hamilton Thorne

- Partial zona dissection

- Zona thinning with acid tyrode’s solution (pH 3.5)
  - Personal communication (A Doyle; TJL)

- Selection of motile sperm, plus removal of cell debris

- All methods require removal of the cumulus cells.
Laser versus normal IVF and Acid Tyrodes IVF

<table>
<thead>
<tr>
<th>Groups</th>
<th>3H1 OOCYTES (n=1)</th>
<th>C3H/HeH OOCYTES (n=3)</th>
<th>129 OOCYTES (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. Oocytes</td>
<td>2-cells</td>
<td>FR%</td>
</tr>
<tr>
<td>Control</td>
<td>2</td>
<td>14</td>
<td>14.1</td>
</tr>
<tr>
<td>Acid Tyrode's</td>
<td>114</td>
<td>33</td>
<td>28.9</td>
</tr>
<tr>
<td>Laser Dissected</td>
<td>39</td>
<td>30</td>
<td>76.9</td>
</tr>
</tbody>
</table>

![Graph showing fertilization rate (%) for different groups](image)

Control
Acid Tyrode's
Laser Dissected

- 3H1 OOCYTES
- C3H/HeH OOCYTES
- 129 OOCYTES
Laser treatment across different backgrounds

- **Fertilization rate (%)**
  - 129S9 (n=3)
  - B6J (n=6)
  - B6NTac (n=11)

- **Birth rate (%)**
  - 129S9 (n=3)
  - B6J (n=6)
  - B6NTac (n=11)
Freezing without cryoprotectants

- Freeze dried sperm stored at $4^\circ$C

- Spermatozoa/spermatids retrieved from reproductive tissues
  - Ogonuki et al (2006) PNAS, 103, 13098

- Freezing in EDTA /Tris-HCL buffered saline

- 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
<table>
<thead>
<tr>
<th>Archiving Summary:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Embryos</strong></td>
<td><strong>Sperm</strong></td>
</tr>
<tr>
<td>Well proven, &gt;35 years</td>
<td>New technology, ~15 years</td>
</tr>
<tr>
<td>Success not particularly strain dependent</td>
<td>Success dependent on genetic background</td>
</tr>
<tr>
<td>Requires large numbers of mice</td>
<td>Only haploid genotype, requires oocytes for IVF</td>
</tr>
<tr>
<td>Requires skilled personnel</td>
<td>Simple, rapid &amp; cheap</td>
</tr>
<tr>
<td>Dissemination is relatively easy</td>
<td>IVF more skilful</td>
</tr>
<tr>
<td></td>
<td>Dissemination more difficult</td>
</tr>
</tbody>
</table>
Genome The End of the Beginning