Harwell Cryopreservation Training Course

MRC embryo archive
Mary Lyon Centre
Harwell Campus, UK
Course aims

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

- Reference point
- Disseminate skills
Mary Lyon Centre – high barrier unit
The MRC frozen embryo archive

- Worldwide Genetic Resource
  - >2850 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)
An international service
Frozen vs live mouse exports
Safety

$\text{LN}_2 = \text{fun}$
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

- Keep samples at LN$_2$ temperature
- Glass transition Temp (Tg) = ~130°C
- Split samples across two or more locations
Data management

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

Responsibility in the use of animals in bioscience research: Expectations of the major research council and charitable funding bodies

Dear [Name],

Date: 30th June 2019

Mouse Identification and Information
Stock code RED/1276
Full nomenclature 129-Eje1w/H-10

<table>
<thead>
<tr>
<th>Animal Identifier</th>
<th>Car</th>
<th>Punch</th>
<th>Date of Birth</th>
<th>Sex</th>
<th>Coat Colour</th>
<th>Genotype</th>
<th>Box No</th>
<th>Compartment No</th>
</tr>
</thead>
<tbody>
<tr>
<td>RED/1276.2f</td>
<td>50</td>
<td></td>
<td>21 February 2010</td>
<td>Female</td>
<td>agouti</td>
<td>wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RED/1278.2h</td>
<td>10</td>
<td></td>
<td>21 February 2010</td>
<td>Female</td>
<td>agouti</td>
<td>Heterozygous</td>
<td></td>
<td>Box 1 Compartment 1</td>
</tr>
<tr>
<td>RED/1279.2g</td>
<td>3</td>
<td></td>
<td>21 February 2010</td>
<td>Female</td>
<td>agouti</td>
<td>wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RED/1278.2i</td>
<td>30</td>
<td></td>
<td>21 February 2010</td>
<td>Male</td>
<td>agouti</td>
<td>Heterozygous</td>
<td></td>
<td>Box 1 Compartment 2</td>
</tr>
<tr>
<td>RED/1278.4j</td>
<td>3</td>
<td></td>
<td>21 February 2010</td>
<td>Male</td>
<td>agouti</td>
<td>wild type</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RED/1276.1n</td>
<td>10+33</td>
<td></td>
<td>21 February 2010</td>
<td>Male</td>
<td>agouti</td>
<td>wild type</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Other Information

Background and origin of strain: 129S6/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 RMD (E) - expanded diet; bedding - Dustwood grade 5 wood shavings; substrate - 'Transgal' water-food supplement for shipment; environmental enrichment - funnix tunnels, paper bedding.

Breeding recommendations: normal breeding regime x 129S6/SvEv

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

An International Centre for Mouse Genetics
EMMA mouse repository
MRC The Mary Lyon Centre
MRC Harwell
Frozen vs live mouse exports
Transport: Dry shippers

- Keep samples at LN$_2$ Temp
- Re-usable
- Considered safe by IATA
- Robust
Transport: unfrozen embryos

• Embryos sent from Madrid to 7 x EMMA nodes
• Refrigerated Temp (4-8°C)
• Luis Montoliu – Madrid (CNB-CSIC)

% embryos recovered

Pups born after transfer

An International Centre for Mouse Genetics

EMMA mouse repository

MRC Harwell
Transport: unfrozen epididymides

- LiFor - organ transport medium  
  (http://www.elimspringsbiotech.com)
- Sphingosine-1-phosphate supplement
- Sperm can be frozen for later use
- MRC and CNB actively using this technique

<table>
<thead>
<tr>
<th>Group (n=3)</th>
<th>No. females used</th>
<th>No. embryos used in IVF</th>
<th>No. 2-cells produced</th>
<th>Fertilisation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72hrs in Lifor -unfrozen</td>
<td>36</td>
<td>1011</td>
<td>751</td>
<td>74.55 ± 1.53</td>
</tr>
<tr>
<td>Lifor- frozen/thawed (24hour)</td>
<td>15</td>
<td>426</td>
<td>356</td>
<td>85.69 ± 4.13</td>
</tr>
<tr>
<td>Lifor- frozen/thawed (48hour)</td>
<td>15</td>
<td>572</td>
<td>320</td>
<td>54.24 ± 7.19</td>
</tr>
<tr>
<td>Lifor- frozen/thawed (72hour)</td>
<td>15</td>
<td>683</td>
<td>115</td>
<td>17.19 ± 2.74</td>
</tr>
<tr>
<td>No Lifor treatment control</td>
<td>18</td>
<td>605</td>
<td>427</td>
<td>66.05 ± 7.70</td>
</tr>
</tbody>
</table>
Transport: sperm on dry-ice

- Marcello Raspa - CNR
Embryo freezing at Harwell
Landmarks in cryopreservation: 1

- 1949: Parkes, Smith & Polge
  - Demonstrated cryoprotective properties of glycerol on fowl sperm
- 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$_2$
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
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, acetamide)
- **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: Some water out
- 5 min: Equilibrium reached
- ProH in
Loading embryos
Embryos frozen in plastic semen straws

- AB12
- Label
- Plug
- Diluent: 1M Sucrose
- Air
- Air
- Embryos in cryoprotectant: 1.5M ProH
- Plug
Seeding the straws
8-cell embryos cooled to -30°C at 0.3 to 0.5°C/min.

Most water out

Ice

Extra-cellular solutes very concentrated
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.
Vitrification

• Numerous protocols exist in the literature e.g. Nakao et al 1997

• DAP213 cryoprotectant - 2M DMSO, 1M Acetamide and 3M Propylene glycol made up in PB1 medium.

• No expensive equipment required

• Advantage - embryos are not subject to chilling injury or blastomere damage from intra/extracellular ice crystal formation.

• Perceived disadvantage – need precise control of the timing of the equilibration steps and the temperature of the solutions.
Embryo/oocyte vitrification

- Method based on DAP213
- Questions
  - Can we apply method to straws?
  - Does equilibration Temp affect survival?
  - How toxic is DAP213?
  - Can oocytes be vitrified successfully?
Vitrification cooling plate

- Aluminium vitrification plate holds samples at 0°C
- Designed at Harwell - Elim Springs Biotech
Simple vitrification protocol

- 1.0M DMSO for 5 mins – 0°C
- DAP213 for 5 mins – 0°C
- Plunge into LN₂
- Thaw in water bath 37°C – this is a critical step
- Equilibrate in 0.9ml 0.25M sucrose
Vitrification procedure - 1

- Prepare cooling platform
Vitrification procedure – 1st step

- Pre-cool 45µl drops of DAP213 and 0.25M sucrose on the VCP
- Transfer all the embryos to a 80µl drop of 1M DMSO, at room Temp.
- Split the embryos into separate drops of DMSO – ready for loading and place them on the VCP for 5 mins
Vitrification procedure – 2nd step

• After 5mins transfer the embryo dish to a microscope
• Use a tapered pipette tip to transfer each group of embryos in 5µl volumes to the 45µl DAP213 drops.
• Start 5 min timer and return embryos to VCP
• After 1min load the straw with 0.25M sucrose, then embryos

• Do this while the embryos are still floating in the DAP213
Vitrification procedure – 3rd step

- Return the straw to the VCP for at least 30sec to re-cool it
- Seal the straw ‘quickly’ and return it to the VCP

- After 5 mins, plunge the straw into LN$_2$
Warming – important step

- Transfer straw to water bath at 37°C
- Hold straw in water until the ice melts
- Remove immediately and wipe dry
- Expel embryos into pre-warmed 0.25M sucrose
## Effect of equilibration Temp.

<table>
<thead>
<tr>
<th>Group</th>
<th># embryo vitrified</th>
<th># embryos found</th>
<th># embryos survived</th>
<th>% survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°C</td>
<td>75</td>
<td>75</td>
<td>73</td>
<td>97.33±1.33</td>
</tr>
<tr>
<td>10°C</td>
<td>75</td>
<td>75</td>
<td>62</td>
<td>80.00±2.31</td>
</tr>
<tr>
<td>20°C</td>
<td>75</td>
<td>75</td>
<td>1</td>
<td>1.33±1.33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th># embryo cultured</th>
<th># blastocyst</th>
<th>% blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>0°C</td>
<td>73</td>
<td>72</td>
<td>98.63±1.39</td>
</tr>
<tr>
<td>10°C</td>
<td>60</td>
<td>52</td>
<td>86.67±2.94</td>
</tr>
<tr>
<td>20°C</td>
<td>1</td>
<td>0</td>
<td>0.00±0.00</td>
</tr>
</tbody>
</table>
### Effect of equilibration time.

<table>
<thead>
<tr>
<th>Group</th>
<th># embryos vitrified</th>
<th># embryos found</th>
<th># embryos survived</th>
<th>% survival ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>5min</td>
<td>90</td>
<td>90</td>
<td>86</td>
<td>95.56±1.11</td>
</tr>
<tr>
<td>10min</td>
<td>90</td>
<td>90</td>
<td>86</td>
<td>95.56±1.11</td>
</tr>
<tr>
<td>20min</td>
<td>89</td>
<td>88</td>
<td>79</td>
<td>88.76±5.66</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th># embryos cultured</th>
<th># blastocyst</th>
<th>% blastocyst ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>5min</td>
<td>86</td>
<td>86</td>
<td>100.00±0.00</td>
</tr>
<tr>
<td>10min</td>
<td>86</td>
<td>79</td>
<td>91.86±6.65</td>
</tr>
<tr>
<td>20min</td>
<td>79</td>
<td>74</td>
<td>93.67±3.06</td>
</tr>
</tbody>
</table>
## Embryo vitrification in straws

<table>
<thead>
<tr>
<th>Group</th>
<th># embryo vitrified</th>
<th># embryo found</th>
<th># embryo survived</th>
<th>% survive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrified in straw</td>
<td>105</td>
<td>105</td>
<td>102</td>
<td>97.14 ± 2.86</td>
</tr>
<tr>
<td>Vitrified in tube</td>
<td>105</td>
<td>103</td>
<td>103</td>
<td>98.10 ± 0.95</td>
</tr>
<tr>
<td>Controlled slow</td>
<td>105</td>
<td>105</td>
<td>96</td>
<td>91.43 ± 3.30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th># embryo cultured</th>
<th># blastocyst</th>
<th>% blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrified in straw</td>
<td>57</td>
<td>54</td>
<td>94.74 ± 2.90</td>
</tr>
<tr>
<td>Vitrified in tube</td>
<td>58</td>
<td>49</td>
<td>84.48 ± 6.16</td>
</tr>
<tr>
<td>Controlled slow</td>
<td>81</td>
<td>70</td>
<td>86.42 ± 1.81</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th># embryo transferred</th>
<th># pups</th>
<th>Birth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrified in straw</td>
<td>30</td>
<td>13</td>
<td>43.33</td>
</tr>
<tr>
<td>Vitrified in tube</td>
<td>30</td>
<td>14</td>
<td>46.67</td>
</tr>
</tbody>
</table>
### Survival rates of vitrified oocytes (C57BL/6J-Tyr<c-Brd>)

<table>
<thead>
<tr>
<th># Oocytes vitrified</th>
<th># Oocytes recovered</th>
<th># Oocytes survived</th>
<th>% survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>171</td>
<td>171</td>
<td>168</td>
<td>98.2%</td>
</tr>
<tr>
<td>99</td>
<td>98</td>
<td>97</td>
<td>99.0%</td>
</tr>
<tr>
<td>80</td>
<td>80</td>
<td>58</td>
<td>72.5%</td>
</tr>
</tbody>
</table>

### IVF fertilisation rates using vitrified oocytes (C57BL/6J-Tyr<c-Brd>)

<table>
<thead>
<tr>
<th>Treatment</th>
<th># Oocytes used</th>
<th># 2 cell embryos</th>
<th>% fertilisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVF using fresh sperm</td>
<td>116</td>
<td>109</td>
<td>94.0%</td>
</tr>
<tr>
<td>IVF using frozen sperm</td>
<td>126</td>
<td>44</td>
<td>34.8%</td>
</tr>
<tr>
<td>Control (Frozen sperm)</td>
<td>142</td>
<td>70.5</td>
<td>49.6%</td>
</tr>
</tbody>
</table>

### Embryos generated from vitrified oocytes (C57BL/6J-Tyr<c-Brd>)

<table>
<thead>
<tr>
<th>Treatment</th>
<th># Embryos transferred</th>
<th># Pups born</th>
<th>Birth rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVF using fresh sperm</td>
<td>15</td>
<td>6</td>
<td>40.0%</td>
</tr>
<tr>
<td>IVF using frozen sperm</td>
<td>18</td>
<td>9</td>
<td>50.0%</td>
</tr>
</tbody>
</table>
Oocyte vitrification – next step

- Huge opportunities for oocyte vitrification
  - Can use the same protocol for embryos/oocytes
  - 50µl volumes of DAP213 allows rapid thawing
    - 0.9ml of warm 0.25M Sucrose
  - Harvest oocytes when colonies are reduced – end of year
  - Rare backgrounds frozen as sperm/oocytes
  - IVFs not restricted by day or time
  - Thaw required number of oocytes
Cryopreservation of mouse sperm

- Another low tech procedure
Sperm freezing equipment
Brief history of mouse sperm cryopreservation

Sperm fertility is influenced by plasma membrane reorganisation, cholesterol sequestration, intracellular Ca\(^{++}\) and reactive oxygen species.

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

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

2008: Ostermeier - 477 \(\mu\)M Mono-thioglycerol (MTG) added to CPA

2009: Liu – AA included in CPA, plus methyl \(\beta\)-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

2014: Nakagata – Rescue protocol for legacy sperm samples
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
- seminal vesicle
- vas deferens
- cauda epididymis
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 vapour
- 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

Aspirate HTF solution

Load the straw

10 x 10µl aliquots of sperm

An International Centre for Mouse Genetics

EMMA mouse repository

MRC Harwell
Sperm freeze method - 3

- Heat seal both ends of the straws
Sperm freeze method - 4

10 minutes

LN₂

h

LN₂
Sperm freezing profile
In vitro fertilisation
Exploitation of *in vitro* fertilisation

- 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
- *In vitro* recombination – soluble Cre
**In Vitro recombination**

- Cell permeable Cre – Excellgen
- 30-40 minute treatment of 2-cell embryos
- 0.3 – 1.8µM final concentration of HTN-Cre
- 70 – 80% efficiency
- Cell permeable Flp - WIP
**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 Ca++(5.14mM) HTF supplemented with 1mM GSH (0.25mM GSH fresh sperm)
- Incubate for 3 to 5hrs, 37°C, 5% CO₂ in air
- Wash eggs, culture overnight in 150μl high Ca++ HTF
- Transfer 2-cell embryos to oviducts of 0.5 day pseudopregnant recipients
The addition of GSH to high Ca\(^{++}\) 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>
Potential of IVF using frozen sperm – (MBCD method)

- 10 x 10\(\mu\)l aliquots of frozen B6N (Sinann, IVF/2605) sperm used to fertilise 209 x B6N oocytes \textit{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
# Legacy sample recovery

<table>
<thead>
<tr>
<th>Group</th>
<th>Genetic Background of sperm</th>
<th>Genetic Background of oocyte</th>
<th>Fertilisation rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FESA</td>
<td>C57BL/6NTac</td>
<td>BALB/cByJ</td>
<td>59.60</td>
</tr>
<tr>
<td>MBCD+GSH</td>
<td>C57BL/6J</td>
<td>C3H</td>
<td>94.30</td>
</tr>
<tr>
<td>FESA</td>
<td>C57BL/6J</td>
<td>C3H</td>
<td>2.11</td>
</tr>
<tr>
<td>MBCD+GSH</td>
<td>C57BL/6J</td>
<td>C57BL/6J</td>
<td>41.13</td>
</tr>
<tr>
<td>FESA</td>
<td>(C57BL/6J x C3H)F1</td>
<td>C57BL/6J</td>
<td>11.13</td>
</tr>
<tr>
<td>MBCD+GSH</td>
<td>(C57BL/6J x C3H)F1</td>
<td>C57BL/6J</td>
<td>35.81</td>
</tr>
<tr>
<td>FESA</td>
<td>B6;129S6/H (Vial)</td>
<td>C57BL/6J</td>
<td>75.00</td>
</tr>
<tr>
<td>MBCD+GSH</td>
<td>B6;129S6/H (Vial)</td>
<td>C57BL/6J</td>
<td>78.30</td>
</tr>
</tbody>
</table>

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
Bio-security

- Most microbial (viral, bacterial & protozoal) agents are removed by ET
- Special cases:
  - Mycoplasma
  - LCMV
  - Parvovirus?
- Wash embryos before transplantation – IETS recommendations
2nd

Genome The End of the Beginning