Transportation of frozen and unfrozen materials

RIKEN BioResource Center
Bioresource Engineering Division
Keiji Mochida
RIKEN BRC (BioResource Center) as a mouse bank

Duties

Collection → Cryopreservation → Distribution

Developer

Live mice → Embryo → Sperm → Cauda epididymides

Mainly preserve as frozen stock

BRC

6,250 strains

Researcher
Archiving Mouse Resources at BRC

- 86% of strains have some genetically artificial modifications.
- 900 inbred strains containing 60 wild-derived strains

BRC has collected unique strains mainly developed in Japan
We distribute mice to **510 organizations** in **32 countries**

in Europe **17 countries**

**International Distribution**

- USA: 37.9%
- China: 12.6%
- Germany: 8.7%
- France: 6.9%
- UK: 4.4%
- Belgium: 3.5%
- Singapore: 3.5%
- Canada: 3.5%
- Italy: 3.5%
- Spain: 3.5%
- Australia: 3.5%

**As of Sep 22, 2011**

Cumulative data since 2001
## List of formats for distribution

<table>
<thead>
<tr>
<th>Format</th>
<th>No. items distributed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live mice</td>
<td>18,217</td>
</tr>
<tr>
<td>Frozen embryos</td>
<td>560</td>
</tr>
<tr>
<td>Recovered litters from frozen embryos</td>
<td>446</td>
</tr>
<tr>
<td>Frozen sperm</td>
<td>32</td>
</tr>
<tr>
<td>Recovered litters from frozen sperm</td>
<td>54</td>
</tr>
<tr>
<td>Recovered litters from FIMRe frozen embryos</td>
<td>6</td>
</tr>
<tr>
<td>Recovered litters from FIMRe frozen sperm</td>
<td>2</td>
</tr>
<tr>
<td>Recovered chimeras from FIMRe ES cells</td>
<td>3</td>
</tr>
<tr>
<td>Only MTA, indirect transfer</td>
<td>121</td>
</tr>
<tr>
<td>frozen or fixed tissues and organs</td>
<td>100</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>14</td>
</tr>
</tbody>
</table>

**FOR 10 Years** 19,555

(Cumulative no. since FY2001)

About 25% of orders were distributed from frozen materials.
**Transportation methods until now**

**Live mice**

- **Advantage**
  1. Possible to use immediately
  2. No need reproductive techniques

- **Disadvantages**
  1. Should keep temperature, fresh air...
  2. Possibilities to die, escape, and spread murine diseases.
  3. Cost of Transportation is expensive

**Dry shipper**

- **Advantage**
  1. Stably keep at under -150°C

- **Disadvantages**
  1. Large and heavy
  2. Expensive
  3. Reproductive techniques are needed
  4. Incurs full fare for round trip
Experiments categorized by temperature

Transportation with

Dry shipper

Embryo → Standard

Sperm → Standard

Experiment 1

Experiment 2

Experiment 3

Experiment 4
Summary of temperature and preservation condition

- 0°C Freezing point of pure water
- -79°C Dry ice temperature
- -110°C to -130°C Glass transition (phase change)
- -150°C Nitrogen gas phase
- -196°C Liquid phase

Danger for storage
Intra-cellular ice formation = Lethal

Safety storage
Transportation with dry-shippers
Preservation in LN₂ tanks
Strategy of novel development

1: Cryopreserve embryos without ice formation.  
2: Using the high concentrated freezing solution.

HOV method
(High Osmolality Vitrification)

Even at -80°C

- No ice formation.
- High survivability.
- Procedures are simple and quick.
Results: After storage at –79°C for 2 days, embryos were survived. But the survival rates were not enough. → defective method!

Exp. 1 Transportation of embryos at -80°C

Embryos: 2-cell stage embryos of C57BL/6J strain
Storage: at -79°C with dry ice pellets for 2 Days
Based vitrification sol.: EFS40 (40%EG, ficoll and sucrose)
Container: 1.2ml cryotube

1. Effect of sucrose concentrations

EFS40 standard Vit. sol.
(Mochida et al. JoVE, 2011)

Conc. of sucrose (mol/l)

Embryos survived

<table>
<thead>
<tr>
<th>Conc. of sucrose</th>
<th>0.3</th>
<th>0.45</th>
<th>0.6</th>
<th>0.75</th>
<th>0.9</th>
<th>10.5</th>
<th>12.0</th>
<th>13.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFS40</td>
<td>32</td>
<td>68</td>
<td>83</td>
<td>78</td>
<td>68</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2. Effect of EG (ethylene glycol) concentrations

Results: Survivability increased over 95%, when embryos were vitrified in solution contains both of sucrose and EG in high concentrations.

Optimal solution was found!
3. Effect of duration at -80°C

Results: Even after storage for 60 and 160 days in deep freezer, over 50% of embryos were survived and developed into offspring.
Exp.1 Transportation of embryos at -80°C

4. International transportation to FIMRe institutes

- MRC Harwell (UK)
- Univ. California Davis (US)
- RIKEN BRC (Japan)

Transport embryos with dryice

LN₂

Dryice package

3 Days

2 Days
### Exp.1 Transportation of embryos at -80°C

#### 5. Results of international transportation to FIMRe institutes

<table>
<thead>
<tr>
<th>Transportation</th>
<th>Recovery method</th>
<th>Transported embryos</th>
<th>Recovery (%)</th>
<th>Normal (%)</th>
<th>Pregnancy (%)</th>
<th>Embryos</th>
<th>Implant. (%)</th>
<th>Offspring (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>Rapid</td>
<td>60</td>
<td>59 (98)</td>
<td>59 (100)</td>
<td>5 / 5 (100)</td>
<td>59</td>
<td>54 (92)</td>
<td>46 (78)</td>
</tr>
<tr>
<td>From Japan to MRC Harwell (UK)</td>
<td>Rapid</td>
<td>75</td>
<td>67 (89)</td>
<td>61 (91)</td>
<td>2 / 2 (100)</td>
<td>43</td>
<td>Not determined</td>
<td>17 (40)</td>
</tr>
<tr>
<td>From Japan to Univ. California Davis (US)</td>
<td>Slow</td>
<td>100</td>
<td>100 (100)</td>
<td>99 (99)</td>
<td>5 / 5 (100)</td>
<td>97</td>
<td>70 (72)</td>
<td>47 (48)</td>
</tr>
</tbody>
</table>

**Results:**

In both institutes, over 90% of transported embryos were morphologically normal, then 40 and 48% of transferred embryos developed into offspring.

Transported embryos in dry-ice package were successfully recovered and developed into healthy mice.
## Exp.1 Transportation of embryos at -80°C

### 6. Survivability of cryopreserved embryos by HOV method in major mouse strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total No. (%) of embryos</th>
<th>No of recipients pregnant/used (%)</th>
<th>No. of embryos</th>
<th>Developed to offspring (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitrified</td>
<td>Retrieved (%)</td>
<td>Alive (%)</td>
<td>Transferred</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>265</td>
<td>263 (97)</td>
<td>256 (97)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>C57BL/6N</td>
<td>175</td>
<td>173 (99)</td>
<td>168 (97)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>BALB/cA</td>
<td>210</td>
<td>210 (100)</td>
<td>206 (98)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>129/SvJ</td>
<td>100</td>
<td>100 (100)</td>
<td>93 (93)</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>DBA/2N</td>
<td>200</td>
<td>200 (100)</td>
<td>193 (97)</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td>C3H/HeN</td>
<td>100</td>
<td>99 (99)</td>
<td>96 (97)</td>
<td>3/3 (100)</td>
</tr>
</tbody>
</table>

**Results:** High survival rates (93-100%) and good ability to develop into offspring (32-82%) in six major inbred mouse strains were confirmed.
Exp. 1 Transportation of embryos at -80°C

7. Procedures of HOV method (optimized for major mouse strains)

(1) Vitrification

Embryos were immersed in equilibrium solution for 3 min.

![Diagram of vitrification process]

- Equilibrium solution: 5% DMSO + 5% EG-PB1, 50 μl
- Vitrification solution: 42.5% EG + 17% Ficoll + 1 M Sucrose-PB1, 50 μl

(2) Liquefy (Slow thawing method)

- Retrieve a tube and let it stand for 3 min.
- Add 850 μl of 0.75 M sucrose-PB1
- Transfer embryos and wash in 50 μl of 0.25 M sucrose-PB1
- Culture in medium until embryo transfer

- Directly immerse in LN₂
- All procedures were performed at room temperature

Don’t need to hurry up! Stable & high results.
Summary of cryopreservation methods for mouse embryo

1. Slow freezing
   - Cooling slowly
   - \(-40^\circ C\)
   - \(-80^\circ C\)

2. Vitrification
   - 3hrs slow freezing
   - Immerse directly into LN\(_2\)

3. Equilibrium slow freezing

4. HOV method
   - More concentrated solution
   - Survive!

Dry ice
- \(-79^\circ C\)
- Lethal

LN\(_2\)
- \(-196^\circ C\)
- Survive!
Exp.2  **Transportation of embryos at 4-8°C**

1. Protocol of transportation developed by Prof. Nakagata in Kumamoto Univ (CARD).

   - **Recovery**: 37°C, 5% CO₂ for 4 hours
   - **Transfer**: into M2 medium
   - **Packing**: with cold pack
   - **Transportation**: 4 °C, 2 days
   - **Transfer into recipient**

2. **Results of domestic transportation**

<table>
<thead>
<tr>
<th>Transportation</th>
<th>Total No. of embryos</th>
<th>No. of tubes</th>
<th>No. of embryos recovered (%)</th>
<th>No. of embryos normal (%)</th>
<th>In vivo development</th>
</tr>
</thead>
<tbody>
<tr>
<td>From CARD to BRC</td>
<td>120</td>
<td>3</td>
<td>120 (100)</td>
<td>120 (100)</td>
<td>pregnant (3/4, 75%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Implantation sites (25/40, 63%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>offspring (16/40, 40%)</td>
</tr>
<tr>
<td>From BRC to NIRS*</td>
<td>96</td>
<td>4</td>
<td>95 (99)</td>
<td>94 (99)</td>
<td>pregnant (5/5, 100%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Implantation sites (55/65, 92%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>offspring (30/60, 50%)</td>
</tr>
</tbody>
</table>

*: National Institute of Radiological Science

**Results:** After transportation for 2 days at refrigeration temperature, most of embryos were morphologically normal. And 40-50% of embryos developed into offspring by embryo transfer. → This method is practically useful.
Exp.3 Transportation of spermatozoa at -80 ℃

1. Summary of transportation with frozen sperm at -80 ℃

(1) Freezing of sperm in 18% raffinose and 3% skim milk solution with plastic straws
   (Takeshima, Nakagata, Ogawa, Exp. Anim. 1991)

(2) Transportation of frozen sperm with dry-ice

(3) Production of live mouse by IVF and embryo transfer

- Add thawed sperm after warming at 37°C for 15 min
- Culture in CZB medium
- BSA-HTF
- Insemination
- 2-cell embryo
- Transfer into recipient
- Offspring
Exp.3  
Transportation of spermatozoa at -80 °C

2. Results of IVF with transported frozen C57BL/6J sperm and development in vivo.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>No. of oocytes</th>
<th>Inseminated</th>
<th>Fertilized (%)</th>
<th>Pregnant (%)</th>
<th>Implantation sites (%)</th>
<th>Offspring (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>137</td>
<td>63 (46.0)</td>
<td>3/3</td>
<td>33/36 (92)</td>
<td>24/36 (67)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>106</td>
<td>60 (56.6)</td>
<td>3/3</td>
<td>34/36 (94)</td>
<td>30/36 (83)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>177</td>
<td>65 (36.7)</td>
<td>3/3</td>
<td>28/36 (78)</td>
<td>24/36 (67)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>183</td>
<td>84 (45.9)</td>
<td>3/3</td>
<td>32/36 (89)</td>
<td>26/36 (72)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>603</td>
<td>46.3 ± 4.1%</td>
<td>12/12 (100)</td>
<td>86.0 ± 4.5%</td>
<td>72.0 ± 3.7%</td>
<td></td>
</tr>
</tbody>
</table>

Results: After transportation with dry-ice for 2 days, we successfully obtained offspring from frozen sperm by in vitro fertilization. 
This transportation procedure is a practical method.

These results were reported at meeting in 2008.
Exp. 3 Transportation of spermatozoa at -80°C

3. Established procedure of IVF with frozen sperm

- Add thawed sperm
- PVA-HTF containing 0.4 mM methyl-
- BSA-HTF supplemented 1 mM GSH*, **
  (reduced glutathione)
- Culture in CZB medium
- 2-cell embryo
- Transfer into recipient
- Offspring

4. Results of IVF with fresh or frozen sperm in standard strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fresh Sperm</th>
<th>Frozen Sperm</th>
<th>Frozen Sperm + GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H/HeJ</td>
<td>74 ± 1</td>
<td>81 ± 1</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>81 ± 1</td>
<td>46 ± 1</td>
<td>75 ± 1</td>
</tr>
<tr>
<td>C57BL/6N</td>
<td>75 ± 1</td>
<td>70 ± 1</td>
<td>79 ± 1</td>
</tr>
<tr>
<td>BALB/cA</td>
<td>93 ± 1</td>
<td>84 ± 1</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>DBA/2N</td>
<td>93 ± 1</td>
<td>87 ± 1</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>129/SvJ</td>
<td>58 ± 1</td>
<td>49 ± 1</td>
<td>58 ± 1</td>
</tr>
</tbody>
</table>

(N = 5-9)

*: P > 0.05

Results: There was no differences between fresh and frozen (added GSH) group except C3H/HeJ strain.
Exp.4  **Transportation of spermatozoa at 4-8°C**

1. Transportation of sperm within epididymides at refrigerated temperature

   - Cauda epididymides
   - 0.5ml Silicone oil
   - Plastic bag
   - 0.8L Thermos

2. Results of IVF after storage of sperm until 5 days
   
   (Mochida, et al. Theriogenology, 2005)

   ![Graph showing 2-cell embryos](chart.png)

   **Results:** Embryos were obtained by IVF with refrigerated epididymides for 5 days, but the rates of fertilization decreased gradually.
Exp.4 Transportation of spermatozoa at 4-8℃

3. Results of IVF after refrigeration of C57BL/6J sperm for 2 days

4. Practical results of IVF after transportation of sperm (C57BL/6J background strains)

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inseminated</td>
<td>70</td>
<td>22</td>
<td>46</td>
<td>10</td>
<td>31</td>
<td>36</td>
<td>70.8 ± 10.0%</td>
</tr>
<tr>
<td>Fertilized</td>
<td>72</td>
<td>24</td>
<td>59</td>
<td>34</td>
<td>50</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>(%)</td>
<td>97.2</td>
<td>91.7</td>
<td>78.0</td>
<td>29.4</td>
<td>62.0</td>
<td>66.7</td>
<td></td>
</tr>
</tbody>
</table>

1mM GSH were added in insemination medium

Results: After transportation of sperm with refrigerated epididymides for 2 days, we successfully obtained embryos even in B6J strain. This transportation procedure has practically used in our center.
1. **Frozen materials**: applicable within 5 days
   - Dry-ice packages for both of embryo and sperm are safe, easy to carry and economical method.

<table>
<thead>
<tr>
<th>Distance</th>
<th>Cost of transportation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live mice (2-3 pairs)</td>
</tr>
<tr>
<td>Domestic (600km)</td>
<td>$200~900</td>
</tr>
<tr>
<td>Intercontinental *</td>
<td>$2,000</td>
</tr>
</tbody>
</table>

*: from Japan to U.S. or Europe

2. **Unfrozen materials**: applicable within 2 days
   - We have often used for only domestic transportation.
   - The refrigeration package is remarkably economical method.

<table>
<thead>
<tr>
<th>Distance</th>
<th>Cost of transportation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Live mice (2-3 pairs)</td>
</tr>
<tr>
<td>Domestic (600km)</td>
<td>$200~900</td>
</tr>
</tbody>
</table>
Conclusion

Efficient transportation methods of embryos and spermatozoa at dry-ice temperature or under refrigeration were devised.

HOV method is eminently applicable for routine embryo cryopreservation in many mouse facilities.

Acknowledgements

We thank
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Please visit our HP

Thank you for your attention !!