



## EMMA Cryopreservation Workshop

CSIC Main Campus, Madrid, Spain

7-8 May 2012

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**International Society for Transgenic Technologies**

The EMMA Cryopreservation Workshop has been organized by EMMA members:

**Martin Fray** (Mary Lyon Centre, MRC, Harwell, UK)  
**Michael Hagn** (Institute of Experimental Genetics, HMGU, Munich, Germany)  
**Lluís Montoliu** (National Center of Biotechnology, CNB-CSIC, Madrid, Spain)

This workshop is sponsored by **EMMA** and co-sponsored by **CSIC** and the **ISTT**

EMMA website: <http://www.emmanet.org>  
WORKSHOP website: <http://www.cnb.csic.es/~criocnb/emma/Madrid2012/>  
ISTT website: <http://www.transtechsociety.org>  
CSIC website: <http://www.csic.es>

## EMMA Cryopreservation Workshop, CSIC, Madrid, Spain, 7-8 May 2012

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## **Welcome to Spain! Welcome to Madrid!, Welcome to CSIC! Welcome to the EMMA Cryopreservation Workshop!**

Madrid, 7 May 2012

Dear Participant,

On behalf of the European Mouse Mutant Archive (EMMA) and the Organizing Committee of this EMMA cryopreservation Workshop, and under the current EU-funded project EMMAService, it is my pleasure to warmly welcome you to Spain, to Madrid, to the CSIC, which will host this EMMA Cryopreservation Workshop on May 7-8, 2012.

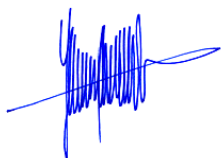
You are at the main Campus of CSIC (*Consejo Superior de Investigaciones Científicas*), the Spanish National Research Council. The CSIC is the largest public institution dedicated to research in Spain and the third largest in Europe. Belonging to the Spanish Ministry of Economy and Competitiveness through the Secretary of State for Research, Development and Innovation, its main objective is to develop and promote research that will help bring about scientific and technological progress, and it is prepared to collaborate with Spanish and foreign entities in order to achieve this aim. The mission of CSIC is to foster, coordinate, develop and promote scientific and technological research, of a multidisciplinary nature, in order to contribute to advancing knowledge and economic, social and cultural development, as well as to train staff and advise public and private entities on this matter.

CSIC research is driven by its centers and institutes, which are spread across all the country, and its more than 15,000 staff, of whom more than 3,000 are staff researchers and about 3,000 are doctors and scientists who are still training. CSIC has 6% of all the staff dedicated to Research and Development in Spain, and they generate approximately 20% of all scientific production in the country. The CSIC has 136 centers and institutions, of which 128 are research institutions (75 wholly CSIC-run centers and 53 joint centers). One of these CSIC research centers is the National Center of Biotechnology, CNB, in Madrid, located at the Campus of the Autonomous University of Madrid in Cantoblanco, and hosting the Spanish node of the EMMA project, officially, since 2009.

At EMMA, we envisage this cryopreservation workshop as a forum to brainstorm and discuss in depth the latest technological advances in cryopreservation, including sperm and embryo cryopreservation, updated IVF methods and related techniques as ovary cryopreservation, laser-assisted and piezo-driven ICSI, transportation of frozen material and other technical and logistic challenges relevant to the operation of current mouse embryo/sperm archives. With this aim, we have invited an extraordinary group of experts in the cryopreservation field, along with delegates from all EMMA nodes and selected members of the International Society for Transgenic Technologies (ISTT), generously co-sponsoring this workshop.

I hope you will truly enjoy participating in this unique workshop on cryopreservation technologies and I also hope you will actively contribute to discussions throughout the meeting. Finally, I strongly recommend you spending some time sightseeing Madrid, its museums and its surrounding beautiful historical cities of Segovia and Toledo, worth visiting at least once in a lifetime.

With my best wishes for a fruitful and productive workshop,



Lluís Montoliu, PhD  
CSIC Research Scientist  
National Center of Biotechnology  
Coordinator of the Spanish EMMA node

## The European Mouse Mutant Archive (EMMA) in May 2012



**EMMA** is a non-profit repository for the collection, archiving (via cryopreservation) and distribution of relevant mutant strains essential for basic biomedical research. The laboratory mouse is the most important mammalian model for studying genetic and multi-factorial diseases in man. Thus the work of EMMA will play a crucial role in exploiting the tremendous potential benefits to human health presented by the current research in mammalian genetics.

The **EMMA network** ([www.emmanet.org](http://www.emmanet.org)) is a partnership of several laboratories and other institutions throughout Europe. The current membership includes the CNR Campus "A. Buzzati-Traverso" in Monterotondo, Italy (core structure), the CNRS Centre de Distribution, de Typage et d'Archivage animal in Orleans, France, the MRC Mammalian Genetics Unit in Harwell, UK, the KI Karolinska Institutet in Stockholm, Sweden, the FCG Instituto Gulbenkian de Ciênciã in Oeiras, Portugal, the HMGU Institute of Experimental Genetics in Munich, Germany, the EMBL European Bioinformatics Institute in Hinxton, UK, the GIE-CERBM Institut Clinique de la Souris, Illkirch, France, the Wellcome Trust Sanger Institute in Hinxton, UK, the CSIC Centro Nacional de Biotecnología in Madrid, Spain, the BSRC "Alexander Fleming" in Vari, Greece, the Institute of Molecular Genetics in Prague, Czech Republic, the UOULU Biocenter Oulu in Oulu, Finland and the VETMEDUNI Biomodels Austria in Vienna, Austria. The EMMA network is directed by Professor Martin Hrabé de Angelis who also heads the HMGU/IEG in Munich. To ensure the operation of such a large and international enterprise an effective management structure consisting of several components was implemented. EMMA is open for the incorporation of new members into the current network to share the increasing workload and guidelines for this process were established.

EMMA is supported by the partner institutions, national research programmes and by the EC's FP7 Capacities Specific Programme. EMMA is currently operating under the EU-project EMMAservice (2009-2012).

## EMMA Cryopreservation Workshop

CSIC, Main Campus, Madrid, Spain

May 7<sup>th</sup> – 8<sup>th</sup>, 2012

[www.emmanet.org](http://www.emmanet.org)

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## WORKSHOP AGENDA

### Day 1 Monday, May 7, 2012

12:00 Reception/lunch/get together at CSIC Students Residence, CSIC Campus

Meeting venue: ICA (*Institute of Agriculture Sciences*) Main Seminar Room, CSIC Campus

#### Day 1: Cryopreservation session

(Chair – Lluís Montoliu)

#### INTRODUCTION, PAST, PRESENT and FUTURE CHALLENGES in CRYOPRESERVATION

13:00 Welcome address (**Lluís Montoliu**)

An outline of EMMA/Infrafrontier (**Michael Hagn**)

13:30 A historical perspective of cryopreservation (**Peter Mazur**)

14:00 Current challenges for bio-repositories: Europe (**Martin Fray**)

[Discussion](#)

14:30 Coffee Break

#### EMBRYO and ORGAN CRYOPRESERVATION

15:00 Controlled rate embryo freezing techniques (**Robert Taft**)

15:20 Vitrification of embryos and oocytes (**Carlisle Landel**)

15:40 Cryopreserving whole organs (**Jorge Sztein**)

[Discussion](#)

#### TRANSPORTATION OF FROZEN AND UNFROZEN MATERIALS

17:00 Transportation of frozen and unfrozen materials (**Keiji Mochida**)

17:20 Shipping sperm frozen samples in dry ice (**Marcello Raspa**)

[Discussion](#) on disseminating materials in the absence of CPA

18:00 [General discussion of all aspects on day 1](#)

19:00 End of day 1

21:00 Dinner (all participants invited)

Restaurant "LA GIRALDA IV" (C. Claudio Coello, 24)

## Day 2 Tuesday, May 8, 2012

### Day 2: New horizons in mouse archiving/recovery and dissemination

(Chair – Martin Fray)

#### SPERM CRYOPRESERVATION AND IVF

08:30 Sperm freezing – the MTG approach (**Michael Wiles**)

08:50 Sperm freezing – the MBCD and GSH approach (**Toru Takeo**)

[Discussion](#)

09:30 Factors affecting IVF (**Sue Bath**)

09:50 IVF using cryopreserved unfertilized oocytes (**Naomi Nakagata**)

[Discussion](#)

10:30 Coffee Break

#### NEW HORIZONS FOR MOUSE GENERATION AND PRESERVATION

11:00 ICSI and its practical applications (**Atsuo Ogura**)

11:20 Novel concepts in mouse production/preservation (**Kent Lloyd**)

11:50 Morula aggregation methods (**Marina Gertsenstein**)

[Discussion](#)

#### OTHER CONTINENTAL EFFORTS TOWARDS CRYOPRESERVATION

12:30 Asia: AMMRA (**Xiang Gao**)

12:40 Australia: APN (**Stuart Read**)

12:50 Canada: CMMR (**Lauryl Nutter**)

13:00 South-America (**Martina Crispo**)

13:10 United States of America: MMRRC (**Kent Lloyd**)

[Discussion](#)

13:30 [Final general discussion](#)

14:30 Lunch/Farewell at CSIC Students Residence, CSIC Campus

## EMMA Cryopreservation Workshop

CSIC, Main Campus, Madrid, Spain

May 7<sup>th</sup> – 8<sup>th</sup>, 2012

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## LIST OF PARTICIPANTS

### Organized by:

**Martin Fray** (Mary Lyon Centre, MRC, Harwell, UK)

**Michael Hagn** (Institute of Experimental Genetics, HMGU, Munich, Germany)

**Lluís Montoliu** (National Center of Biotechnology, CNB-CSIC, Madrid, Spain)

### Invited speakers:

**Sue Bath** (Melbourne, Australia)

**Martina Crispo** (Institut Pasteur, Montevideo, Uruguay)

**Xiang Gao** (Model Animal Research Center, Nanjing University, Nanjing, P.R. China)

**Marina Gertsenstein** (Toronto Centre for Phenogenomics, Toronto, ON, Canada)

**Carlisle Landel** (Transposagen Biopharmaceuticals, Inc., Lexington, KY, USA)

**Kent Lloyd** (Mouse Biology Program, University of California, Davis, CA, USA)

**Peter Mazur** (The University of Tennessee, Knoxville, TN, USA)

**Keiji Mochida** (RIKEN Bioresource Center, Tsukuba-shi, Ibaraki, Japan)

**Naomi Nakagata** (Center for Animal Resources & Development-CARD, Kumamoto University, Kumamoto, Japan)

**Lauryl Nutter** (The Hospital for Sick Children, Toronto, ON, Canada)

**Atsuo Ogura** (RIKEN Bioresource Center, Tsukuba-shi, Ibaraki, Japan)

**Marcello Raspa** (CNR-EMMA, Monterotondo/Rome, Italy)

**Stuart Read** (The Australian National University, Australian Phenome Bank, Canberra, Australia)

**Jorge Sztejn** (CMB Cryopreservation and Assisted Reproduction, NIAID-NIH, Rockville, MD, USA)

**Rob Taft** (The Jackson Laboratory, Bar Harbor, ME, USA)

**Toru Takeo** (Center for Animal Resources & Development-CARD, Kumamoto University, Kumamoto, Japan)

**Michael Wiles** (The Jackson Laboratory, Bar Harbor, ME, USA)

### Invited participants:

**Alan Hart** (MRC-Human Genetics Unit at MRC/IGMM, University of Edinburgh, Edinburgh, UK)

**Jean Jaubert** (Institut Pasteur, Paris, France)

**Pedro Moreira** (European Molecular Biology Laboratory, Monterotondo/Rome, Italy)

**Sagrario Ortega** (National Cancer Research Center, CNIO-ISCIII, Madrid, Spain)

**Belén Pintado** (National Center of Biotechnology, CNB-CSIC, Madrid, Spain)

**Xavier Warot** (Center of PhenoGenomics, School of Life Sciences, EPFL, Lausanne, Switzerland)

**Isabelle Barde** (Center of PhenoGenomics, School of Life Sciences, EPFL, Lausanne, Switzerland)



Delegates from EMMA nodes: <http://www.emmanet.org>

**Thomas Ruelicke** (VETMEDUNI/BIAT, Vienna, Austria)  
**Auke Boersma** (VETMEDUNI/BIAT, Vienna, Austria)  
**Amanda Pickard** (Mary Lyon Centre, MRC, Harwell, UK)  
**Mo Guan** (Mary Lyon Centre, MRC, Harwell, UK)  
**Kostas Bozonelos** (BSRC "Alexander Fleming", Vari/Athens, Greece)  
**Susan Marschall** (HMGU/IEG, Munich, Germany)  
**Sabine Fessele** (HMGU/IEG, Munich, Germany)  
**Yi Hong** (HMGU/IEG, Munich, Germany)  
**Richard Houghton** (WTSI, Hinxton, UK)  
**Johannes Wilbertz** (KI, Stockholm, Sweden)  
**Hanna Karlsson** (KI, Stockholm, Sweden)  
**Raija Soininen** (UOULU/BCO, Oulu, Finland)  
**Inken Beck** (IMG, Prague, Czech Republic)  
**Ferdinando Scavizzi** (CNR, Monterotondo/Rome, Italy)  
**Brendan Doe** (CNR, Monterotondo/Rome, Italy)  
**Abdelkader Ayadi** (GIE-CERBM/ICS, Illkirch/Strasbourg, France)  
**Philippe André** (GIE-CERBM/ICS, Illkirch/Strasbourg, France)  
**Joana Bom** (IGC, Oeiras, Portugal)  
**Ana Sofia Leocádio** (IGC, Oeiras, Portugal)  
**María Jesús del Hierro** (CNB, CSIC, Madrid, Spain)  
**Julia Fernández** (CNB, CSIC, Madrid, Spain)

Selected participants from the ISTT:

<http://www.transtechsociety.org>

**Jan Parker-Thornburg** (The University of Texas-MD Anderson Cancer Center, Houston, TX, USA)  
**Gonzalo Moreno del Val** (Institute of Neurosciences, CSIC-UMH, Alicante, Spain)  
**Rubina L. Caldeira dos Santos** (Champalimaud Centre for the Unknown, Lisbon, Portugal)  
**Anna Crowley** (University College London, UK)  
**Hans-Christian Theussl** (Research Institute of Molecular Pathology IMP/IMBA, Vienna, Austria)  
**Aimee Stablewski** (Roswell Park Cancer Institute, Buffalo, NY, USA)  
**Rada Norinsky** (Rockefeller University, New York, NY, USA)  
**Mary Ann Haskings** (London Research Institute, Cancer Research UK, Potters Bar, Hertfordshire, UK)  
**Rebecca Haffner** (Weizmann Institute of Science, Rehovot, Israel)  
**Jaime Muñoz** (Spanish National Cancer Research Centre, CNIO, Madrid, Spain)  
**Judith Fiedler** (Max-Planck-Institute for Molecular Genetics, Berlin, Germany)  
**Steven Sansing** (Charles River Laboratories, Wilmington, MA, USA)

## **ABSTRACTS prepared by INVITED SPEAKERS**

(listed in order, according to the program of this EMMA cryopreservation workshop)

## EMMA – The European Mouse Mutant Archive

**Michael Hagn**

Institute of Experimental Genetics, HMGU, Munich, Germany

EMMA's primary objectives are to establish and manage a unified repository for maintaining mouse mutations and to make them available to the scientific community. In addition to these core services, the consortium can generate germ-free (axenic) mice for its customers and also hosts courses in cryopreservation. The EMMA network is currently comprised of 14 partners who operate as the primary mouse repository in Europe. Israel and Netherlands will join the EMMA network in 2012 as new member states. EMMA is funded by the participating institutes and the European Commission FP7 Capacities Specific Program. The EMMA network operates on a partial cost recovery model and offers the scientific community a free archiving service for mutant mouse lines and access on a fee- for-service basis to a wide range of disease models and other research tools. The EMMA archive currently comprises about 3000 mouse mutant lines that were either submitted by individual investigators or contributed by EU funded projects such as EUCOMM and EUMODIC. EMMA's mouse mutant resources are widely demanded and at present nearly 600 mouse mutant lines are shipped annually to customers around the world. Drivers of a continued growth of the EMMA archive are large scale programs and research projects such as the International Mouse Phenotyping Consortium, the Sanger Mouse Genetics Project, EUCOMMtools and the emerging Collaborative Cross resource. These developments necessitate continued efforts in technology development, capacity building and securing sustainable funding.

These issues are being addressed by the Infrafrontier consortium, which is part of the ESFRI Roadmap since the year 2006. Infrafrontier combines the European mouse clinics and the EMMA network. Infrafrontier objectives are:

1) Building of sufficient capacities for systemic phenotyping archiving and distribution. These scientific platforms and services will be accessible for both, individual research projects using mice of different genetic resources and for large-scale programs such as the IMPC;

2) Securing sustainable funding for the mouse productions centres, mouse repositories and primary phenotyping centres that contribute to the Infrafrontier Research Infrastructure;

3) Providing a single point-of entry for the users of the scientific platforms and services offered by the Infrafrontier Research Infrastructure underpinned by common quality standards and operation procedures and a pan-European capacity and risk management.

The Infrafrontier consortium consists of 29 partners, representing the leading mouse clinics and archiving and distribution nodes, and the related ministries and major funding bodies from 12 European countries and Canada. The pan-European activities of the Infrafrontier Research Infrastructure will be coordinated by the Infrafrontier Legal Entity, which is currently being set up by the European member states as a German private limited company.

The Infafrontier partners very recently secured a 9.9 Mill EUR EC grant that aims to meet the future challenges presented by phenotyping, archiving and disseminating mouse models in the European Research Area as follows: 1) Contribute to resource development by archiving of 1215 new mouse mutant lines; 2) Provide free of charge Transnational Access to mouse production and 1st line phenotyping capacities; 3) Offer a specialized axenic service to produce, maintain and to distribute germ-free mice; 4) Provide user friendly accession of Infrafrontier services, extensive manual data curation and cross referencing with other mouse database; 5) Improve user services by developing novel phenotyping and cryopreservation SOPs and by refining innovative research instrumentation; 6) Engage with the user community using a wide range of PR activities, a dedicated user meeting and an industry liaison workshop; 7) Offer state of the art cryopreservation and phenotyping training courses.

## The Cryobiology of Mammalian Oocytes and Embryos— Past, Present, and Future

Peter Mazur

Department of Biochemistry and Cellular and Molecular Biology, The University of Tennessee, Knoxville, USA

Although bovine and human sperm were successfully cryopreserved in 1960 using slow cooling and glycerol as the cryoprotective agent, it took another 12 years to successfully preserve a mammalian embryo. In 1963, I had carried out and published physical-chemical analyses that proposed that if cells were cooled too rapidly, they would undergo intracellular ice formation (IIF) with probable lethal consequences, and defined what is meant by “too rapidly” for a particular cell. To avoid IIF, the cell had to dehydrate during cooling, and the required rate of cooling depended chiefly on the permeability of a particular cell to water and on the temperature coefficient of that permeability.

Between 1968 and 1972, Stanley Leibo and I turned our attention to the survival of yeast, mouse marrow stem cells, and Chinese Hamster tissue culture cells as functions of cooling rate and temperature. We found that plots of survival vs. cooling rate formed an inverted “U”, with high survivals at a medium rate, and lower survivals after very slow and very rapid cooling. We proposed a “Two-Factor” hypothesis which stated that the right hand arm of the inverted U was a result of IIF at high cooling rates. Interestingly, the optimum cooling rate for maximum survival varied over a wide range for the different cells. This was chiefly because the cells differed widely in water permeability and the temperature coefficient of that permeability. In fact if the known values of these parameters were used to generate the theoretical curves, the predicted relation between IIF and cooling rate agreed quite closely with the observed drop in survival vs. cooling rate.

In 1971 David Whittingham reported the successful cryopreservation of mouse 8-cell embryos in PVP and cooled at 50°C/min. Leibo and I were excited but perplexed about these results— Perplexed because our computations indicated that because of their large size, mouse embryos would undergo lethal IIF when cooled at 50°C/min. Our calculations indicated that the cooling rate would have to be about 1°C/min to avoid IIF. In Spring 1972, we invited David over, We were unable to repeat his results using his media. But when we slowed the rate to 1°C/min and substituted DMSO for PVP, we obtained very good in vitro and in vivo survival of 8-cell embryos cooled to -196°C (or -269°C). The ability to cryopreserve mouse embryos quickly had important applied consequences and important fundamental consequences. Perhaps, the most important direct application was that it permitted the long-term maintenance of thousands of mutant lines of mice in the form of preimplantation embryos in a few liquid nitrogen tanks. The indirect applications stemmed from the fact that essentially the same procedures worked for early embryos of over 20 other mammals, including humans.

In the last decade, clinical interest has been emphasizing the cryopreservation of human oocytes. They have proved more difficult to freeze and so interest has shifted somewhat to cryopreserving them by vitrification. As discussed, one way to avoid lethal IIF is to cool cells slowly enough so that the freezable water in the cell flows out of the cell and freezes externally. An alternative approach is to avoid intracellular ice by vitrifying cellular water. Vitrification involves converting water into a non-crystalline or amorphous glass. To achieve this conversion, it has been the strong belief that cells have to be in solutions containing a very high concentration of glass-inducing solutes and have to be cooled very rapidly. In recent work, however, we have found that neither of these is true for mouse oocytes. The important factor is not the cooling rate—It is the warming rate, and the warming rate has to be very high. Furthermore, if the warming rate is very high, one can cut in half the concentration of solutes in the vitrification solution and still obtain high survivals.

## Current challenges for bio-repositories

Mo Guan, Amanda R. Pickard & **Martin D. Fray**

Mary Lyon Centre, Medical Research Council, Harwell Science and Innovation Campus, Oxfordshire, OX11 0RD, UK

The activities of individual laboratories and large-scale mutagenesis programmes around the world are generating huge numbers of novel GA mouse strains that need to be securely archived. Moreover, the construction of extensive research infrastructures for systematic phenotyping, such as EUMODIC and the IMPC is fuelling demand for these GA mice. Consequently, the principal challenges facing all mouse repositories centre on ensuring they have secure long term funding that will enable them to satisfying future demand for both archiving and dissemination services.

In order to meet the demand for archiving services, cryopreservation techniques needed to be continually refined to reduce animal usage and increase throughput. Unfortunately, strains carrying complex genetics will still require freezing as embryos but new vitrification procedures could accelerate this process. Fortunately, over the last 5 years there have been some significant advances in sperm freezing technologies. It is now common place to archive mouse strains by freezing the sperm harvested from 4 or so males. This means that repositories need to focus on their in vitro fertilisation and assisted reproduction procedures to ensure mouse strains can be recovered efficiently. This includes having access to robust ISCI skills, enabling repositories to virtually guarantee recovering specific mouse lines if male germplasm is available. This concept should be extended to include the recovery of live born pups from germ cells harvested from testicular tissue or sperm frozen in the absence of cryoprotectants and stored in conventional laboratory freezers.

Moreover, new approaches to the distribution of mouse stocks need to be developed in order to reduce the dependency on the transportation of live mice and to reduce the cost of transportation. Both embryos and spermatozoa can maintain their viability ex vivo for several days if held at temperatures between 4 to 8°C, permitting shipment in chilled containers. What is more, frozen embryos and sperm can be held on dry ice for several days and still be used to generate live born pups. Repositories need to embrace these technologies so they can eliminate the cost of transporting dry-shippers when embryos and sperm are exchanged.

Repositories also provide a valuable service to smaller laboratories that want to protect/distribute their mouse lines, which presents the repositories with new challenges. Few would argue that the GA mice generated through publicly funded research shouldn't be accessible to the wider scientific community. Nevertheless, there are both real and perceived barriers to sharing e.g. the reluctance of scientists to release a strain which they have generated. Archive managers need to work hard to persuade individual scientists to deposit their stains in secure, public repositories and share them freely with the community. In doing so, they will increase the scientific value of the archive. Another barrier to sharing can be the desire to retain intellectual property rights. Executing an MTA can be real hindrance to the speed stocks can be released to the community. This is particularly troublesome when interacting with for-profit organisations who find it difficult to accept some of the restrictive MTAs in existence today. This is a real impediment to translational research and depositors need to be persuaded not to attach overly restrictive MTAs to the mouse lines they generate.

Another significant barrier to exploiting the resources held within repositories is a lack of knowledge on what is available to share, where the information can be found and how to handle the materials available for dissemination. This is despite the development of searchable databases such as the International Mutant Strain Resource (IMSR: [www.findmice.com](http://www.findmice.com)) and the portals maintained by individual repositories. It is, therefore, necessary to continually educate researchers about the resources available to them and how they should be handled. In essence, this means maintaining a network of cryo-recovery training courses which show people how to handle the materials held within their archives. By addressing these challenges, repositories will ensure they continue to provide the high level service necessary to under pin future developments in the biomedical sciences.

## Controlled rate embryo freezing

**Rob Taft**

The Jackson Laboratory, Bar Harbor, ME, USA

Forty years ago Whittingham, Leibo and Mazur reported that after exposure to dimethyl sulfoxide and cooling at a rate of 0.2-3.0 degrees per minute, 50-70% of mouse embryos survived freezing and thawing and that after transferring embryos into pseudopregnant recipients "more than 40% of the embryos in these pregnant mice gave rise to normal, living full-term fetuses or newborn mice". This work helped launch a new field and has been the foundation for protocols used successfully to cryopreserve embryos from many species of mammals, impacting laboratory animal research as well as animal agriculture and human medicine.

Today, controlled rate embryo freezing is probably the most widely used method for cryopreserving mouse embryos and it is highly effective as embryo survival is typically well over 90%. At The Jackson Laboratory, we have cryopreserved more than 3.0 million mouse embryos from thousands of strains and have found controlled rate embryo freezing to be effective, robust and reliable. We have also found it well suited for use in a high-throughput operation as batches of embryos can be frozen simultaneously, simplifying quality control and reducing person-person variability.

A limitation of controlled rate embryo freezing has been the cost of purchasing a controlled rate freezer, but until 1985 there were no alternatives. In 1985 Rall reported that mouse embryos could be vitrified, eliminating the need for a controlled rate freezer. Interest in vitrification grew following this report, resulting in many attempts to compare controlled rate embryo freezing and vitrification. The question of which approach is "better" has not been definitively answered and may not need to be. Core facilities setting up cryopreservation programs or assessing their cryopreservation programs can choose among effective controlled rate embryo freezing protocols and embryo vitrification protocols, picking approaches that best fit with their scale, workflow and budget.

Globally, interest in reducing animal usage increasing and pressure to contain costs is rising. With multiple effective protocols to choose from, reducing cost and animal use are becoming increasingly important. Embryo yield is relatively low per female and embryo yield as well as embryo developmental capacity vary widely among strains. Consequently, cryopreserving embryos can be expensive, time consuming and require the use of many animals. Dedicating effort to reducing the time, cost and number of animals required for cryopreserving strains will help ensure that the full value of cryopreservation as a tool for managing colonies and facilitating distribution is realized in the future.

## Vitrification of embryos and oocytes

**Carlisle P. Landel**

Transposagen Biopharmaceuticals Inc., Lexington, KY, USA

This talk will focus on the background, current methodology, and potential pitfalls and advantages of so-called vitrification methods for the cryopreservation of mouse embryos and oocytes. Vitrification provides an alternative to equilibrium methods for cryopreserving germplasm. In this approach, samples are cooled at rates so rapid that the solution forms a glass rather than a crystalline solid. Since it is the formation of intracellular ice crystals that is inimical to the survival of the cryopreserved cell, vitrification provides a method to circumvent this disruptive effect. Vitrification methods are being successfully utilized for the cryopreservation of mouse (and other mammalian) germplasm. In general, these methods are simple, rapid and easy, but samples created through vitrification must be stored and handled in a manner that avoids the glass transition, in which the vitrified sample spontaneously converts to a crystalline solid, i.e., ice crystals form, destroying the cryopreserved cells. Since this transition occurs at temperatures well below the freezing temperature of the solution ( $\sim -130^{\circ}\text{C}$ ), care must be taken in the proper storage and recovery of vitrified samples.

## Cryopreservation of Organs

**Jorge M. Sztejn**

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The idea of cryopreserving reproductive organs was conceived around 1780 when L. Spallanzani<sup>1</sup> realized that frog sperm could be maintained longer in cold conditions. Later Mantegazza<sup>2</sup> continued those sperm studies and tried to perform a "Therapeutic Transfer" or what we call today the artificial insemination. However, these thoughts became stronger in 1949 when Polge, Smith and Parkes<sup>3</sup> discovered glycerol. In the earlier 50's Audrey Smith and James Lockwood<sup>4</sup> discovered that a slow freezing to -79 °C in a solution containing 15% glycerol followed by a rapid warming works for many cells, a discovery, which was the precursor of the programmed rate freezer we use today. David Whittingham, S Leibo, and P Mazur<sup>5</sup> utilizing a slow freezing method achieved the first mouse embryo freezing in 1972. Following that milestone not only cryobiology was established as an entity, but many concepts were proven too. Among those principles, we learn from Peter Mazur<sup>6</sup> about the speed of freezing and the consequences of the intracellular ice formation, and from Staley Leibo<sup>7</sup> that each cell type has their unique freezing curve. All those new concepts make it rational of how difficult it is to freeze an organ structured by multiple tissues with different architecture and different function. However, the empiric science shows the contrary. Parrot<sup>8</sup> in 1960 described the ovary freezing followed by an orthotopic transfer, although no pups were born, the author described that the ovary cannot be stored for more than 44 days. The general concept presumes that the cryo-injury affects the ovarian follicles. During the 90's few groups were working in developing a reliable technique for freezing the ovary. At the Jackson Laboratory a slow freezing technique using DMSO as cryoprotectant (CPA) was developed using slow freezing till -80 with very good results<sup>9</sup>. This study was followed by a complete histopathology of the frozen-thawed ovary which, indeed, showed that only the large follicles were affected, but the immature oocytes survive the process and produce fertile ova. An in vitro maturation of the frozen ovary followed by IVF to obtain live pups was also done to prove that the immature oocytes survived the freezing<sup>10</sup>. In 2009 the freezing technique was modified to a two-step freezing in straws using PROH as CPA which has improved the results after surgically transferring the ovary pieces<sup>11</sup>.

With respect to testicular tissue the problem encountered is that there is no reliable surgery to reestablish the fully function of the organ. The reported orthotopic surgery only sustains the spermatogenesis, but need help of ancillary techniques like ICSI to achieve fertility.

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## Transportation of frozen and unfrozen materials

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In recent years, the number of available genetically modified mouse strains has increased greatly. As sharing these valuable genetic resources, mice are frequently transferred from one facility to another. However, the transportation of live mice is costly, and there is an increased risk of spreading murine diseases. Therefore, the transfer of cryopreserved embryos and spermatozoa is the method currently preferred. We have developed four new protocols for the transport of frozen and unfrozen embryos and spermatozoa at dry-ice and refrigerated temperature.

First, we have shown that when using a novel high osmolality vitrification (HOV) method, 2-cell embryos produced by IVF can be stored at dry-ice temperature ( $-79^{\circ}\text{C}$ ) for more than 160 days with high survival rates. Such embryos have been successfully transported domestically and internationally; from RIKEN BRC to the UC Davis, and to the MRC in Harwell. The HOV method employs a concentrated cryoreagent (about 35 mol/Kg water), and using this simple protocol we have demonstrated high rates of embryo survival (93–100%) and offspring development success (33–82%) in six major inbred mouse strains. In a second protocol, developed at Kumamoto University (CARD), by Professor Nakagata, 2-cell embryos were transferred into M2 medium in 0.5-ml plastic tubes, and transported under refrigeration. With this procedure, 100% (120/120) of C57BL/6J embryos survived for 2 days during transfer from CARD to RIKEN BRC and were morphologically normal. Moreover, with these embryos, 40% of those transferred developed into offspring.

Third, frozen C57BL/6J spermatozoa were cryopreserved (in 18% raffinose and 3% skim milk) inside a plastic straw and transported at dry-ice temperature from CARD to RIKEN BRC. Using these sperm, 46% ( $\pm 10.1\%$ ,  $N = 4$ ) of C57BL/6J oocytes were fertilized in HTF medium supplemented for the preincubation period with methyl- $\beta$ -cyclodextrin (MBCD; 0.4 mM) and hypotaurine, and 72% of the transferred embryos developed into live offspring. In a fourth protocol, we have shown that C57BL6J spermatozoa can be transported under refrigeration within epididymides. When using spermatozoa retrieved from refrigerated ( $6-8^{\circ}\text{C}$ ) epididymides for 2 days, 71% of oocytes were fertilized successfully in vitro with the fertilization medium containing 1 mM reduced glutathione, additionally. We are currently applying this protocol within our center for the collection of many different mouse strains.

Thus, efficient transportation methods of embryos and spermatozoa at dry-ice temperature or under refrigeration were devised. We propose that the HOV method for embryo cryopreservation will be particularly useful for many facilities involved in transportation of mice.

## Shipping sperm frozen samples in dry ice

**Marcello Raspa**

Consiglio Nazionale delle Ricerche - European Mouse Mutant Archive (CNR-EMMA). Institute of Cell Biology and Neurobiology. Campus A. Buzzati-Traverso, Monterotondo Scalo, Roma, Italy

Recent progress in molecular genetics and developmental biology have created thousands of newly engineered strains of laboratory animals, mostly rodents (GA-mice), drosophila, and zebrafish. This process, now irreversible, will in fact accelerate its pace. The mouse shows great similarities in development, physiology and biochemistry to humans. This makes it a key model for research into human disease. Following the Mouse and Human gene sequencing and analysis (2001 & 2002) large-scale mouse knockout programs are producing a permanent resource of targeted mutations in all protein-coding genes. Thousands of GA mice will be generated and distributed in the coming years to the wider research community. To fully exploit these emerging resources, as developed by international initiatives such as the International Knock-Out Mouse Consortium (IKMC), mouse models must be cryo-preserved and made readily available to the international biomedical research community. Moreover, the establishment of distributed and collaborative research infrastructures for systemic phenotyping, archiving and distribution will further increase the need to move mouse strains around the world. Disseminating mouse stocks as frozen materials offers ethical, logistical and economical advantages over live animal shipment, minimizing the welfare issues and avoiding some of the complex customs regulations that are associated with live animal transportation. Considering the above issues and the imperative to attain the highest welfare standards, every effort should be made to disseminate mouse strains as embryos, reproductive tissues or germplasm, whenever it is practical to do so. The current preference for importing stocks as live mice is of course influenced by the traditional, better known animal handling procedures and the recipient's inability to properly handle frozen materials. Maintaining a continuing program of training for disseminating the most innovative tools on mouse cryopreservation will therefore help on educating lab personnel and professional leaders in the methods needed to handle frozen stocks. Embryo freezing has traditionally been the method of choice for archiving mouse lines while sperm freezing is emerging as modern alternative due to the application of innovative protocols. A major advantage of sperm freezing is that it is easier for beginners and does not require any expensive equipment. Moreover it greatly enhances the ability to rapidly and economically archive the increasing number of mutants that are being generated by international large-scale mutagenesis programs. Frozen sperm are less sensitive to post-freezing temperature fluctuations but have traditionally been transported using dry-shippers. We show the reliability of distribution of frozen sperm samples stored in dry ice, through a pilot study conducted among four EMMA partners (Consiglio Nazionale delle Ricerche, Centro National de Biotecnologia, Helmholtz Zentrum Muenchen and Medical Research Council). The experimental data show that it is possible to produce 2-cell embryos and offspring from sperm permanently kept in dry-ice and comparable efficiencies are obtained after subsequent refreezing in liquid nitrogen. From the practical point of view this protocol provides sufficient stability to make it feasible to ship sperm samples worldwide in dry ice. Recent developments involve the transportation of embryos on dry ice following vitrification at near-equilibrium freezing and they have been shown to withstand an extended period of storage (up to 4 days at  $-80^{\circ}\text{C}$ ). There appears to be no limit on the shipping times, as long as the dry-ice container can be kept topped up. The other advantage of this procedure is that the shipped straws can be placed in at  $-80^{\circ}\text{C}$  freezer on arrival or re-plunged in LN2 without significant alteration to the sperm viability. This makes coordinating the shipment date with the availability of embryo recipient females less critical. Further studies are still in progress aiming to check the viability of mouse sperm maintained at  $-80^{\circ}\text{C}$  for a medium period of storage.

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## Mouse Sperm Cryopreservation at JAX

**Michael V. Wiles**

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In 1909 Clarence Cook Little developed the first inbred mouse strain, since then the number of strains has steadily increased. With the advent of genetic engineering in recent years, the number of mouse strains has exploded. These strains are essentially tools designed to answer a variety of questions. As these questions are addressed and answered the fate of each individual strain is in the balance; i.e. keep or kill ! Maintaining strains as live colonies is financially draining and carries the continual risks of a disastrous loss due to fire, flood, disease etc, or simply breeding cessation - hence the need to cryopreserve. Storage by embryo cryopreservation is well established however, the production of sufficient embryos is expensive. In contrast sperm cryopreservation is potentially cheap, although it can only preserve the paternal lineage. A major obstacle was that sperm cryopreservation was the extreme variations in success between major backgrounds. Recently at JAX we devised an economic general process which is applicable to all major inbred background and can be carried out in a production lab environment. It is also simple, making it accessible to individual labs via e.g. a sperm cryo kit.

The process uses an established sperm cryopreservation buffer of raffinose and skim milk, but crucially includes monothioglycerol. Sperm is frozen and stored in 250  $\mu$ l French straws at high density in liquid nitrogen. The subsequent thaw/IVF process is also simple, with sperm being merely thawed and incubated in MZF media for 1hr prior adding freshly isolated oocytes. This approach works well for most of the commonly used background, including importantly C57BL/6, which routinely gives  $\geq 50\%$  recovery to two cell stage post IVF.

Our main driver and measure of success is scale - for example over the last 3-4 years using this approach we have cryopreserved (and QC'ed) sperm from  $\sim 6,700$  mouse strains. In the last year alone we froze  $\sim 2100$  strains, and estimate that we recovered  $\sim 1500$  strains in the same period; i.e. it works.

The question open to discussion here is, can we make better sperm cryopreservation buffers, e.g. using, for example other antioxidants ? - Undoubtedly, yes. However, do we need to ? Currently the biggest losses in the process are the loss of animals from two cell to live born .. why is this?

At this meeting we will review what JAX has done, the future challenge as seen from a Production Lab's perspective, and address our future challenges, including more strains, complex backgrounds (e.g. the CC), the cryopreservation of strains carrying multiple genetic modifications, outbreeds (e.g. the DO) and perhaps the Future Shock of mice made to order and what this means to cryo-storage and recovery.

## Sperm freezing – the MBCD and GSH approach

**Toru Takeo**

Center for Animal Resources and Development (CARD), Kumamoto University, Japan

Mouse sperm cryopreservation is an essential technology for the creation of an efficient mouse bank system. Since 1990, many papers have been published on the topic of improving the technology used in mouse sperm cryopreservation and in vitro fertilization (IVF) using frozen-thawed sperm. In 2000, Nakagata et al. developed a standard protocol for mouse sperm cryopreservation, known as the Nakagata method. This method ensures high sperm viability and fertility in most inbred and hybrid strains of mice. However, the method is unable to sufficiently protect the sperm of C57BL/6 mice, a major inbred strain used for producing genetically engineered mice. This is due to C57BL/6 sperm being highly sensitive to the stresses of freezing and thawing. In mouse banks, the low fertility of frozen-thawed C57BL/6 mouse sperm was a serious problem when attempting to use cryopreserved sperm derived from genetically engineered mice based on the C57BL/6 strain for reproduction with live mice via IVF.

To overcome this problem, we examined the causes of fertility reduction in cryopreserved C57BL/6 mouse sperm, and made changes and improvements to sperm cryopreservation and IVF in order to enhance the fertility of frozen-thawed C57BL/6 mouse sperm. Firstly, we modified the cryopreservation procedures and the cryoprotectant (18% raffinose pentahydrate/3% skim milk solution: R18S3). This is because we found that adding amino acids, especially L-glutamine, to R18S3 improved the fertility of frozen-thawed sperm. Secondly, we discovered that methyl- $\beta$ -cyclodextrin (MBCD) greatly increased the fertilization ability of the sperm by facilitating cholesterol efflux from the cells during sperm preincubation. Thirdly, we found that treating oocytes with reduced glutathione (GSH) cleaved disulfide bonds in the zona pellucida (ZP) and induced morphological changes in the ZP, resulting in easy sperm penetration through the ZP.

In summary, our optimized protocol for sperm cryopreservation and IVF provides a high and stable recovery rate for frozen-thawed C57BL/6 mouse sperm.

## Factors affecting in vitro fertilization using cryopreserved mouse sperm

**Sue Bath**

Melbourne, Australia

Intrinsic (effect of strain on eggs and sperm) and extrinsic (methodologic) factors affecting in vitro fertilization (IVF) will be reviewed briefly. Over the past ten years, IVF rates using frozen-thawed C57BL/6J sperm have increased from less than 10% to above 60%. This is the result mainly of improvements in the IVF protocol, not in the cryoprotectant (CPA). Determining whether one CPA is better than another is complicated, because deficiencies in a CPA can be overcome by the IVF procedure, which usually is not standardized among studies. Interpretation of apparent improvements in CPA has been hampered by failure to use an increase in fertilization rate as the criterion to confirm better post-thaw fertility.

Data will be presented comparing IVF using sperm frozen in Fertipup CPA with IVF using sperm frozen in 23.7% raffinose. Post-thaw fertility is improved by excluding immotile/damaged sperm from the fertilization milieu, achieved by reducing the concentration of the sperm; by selecting motile sperm to fertilize the eggs; by washing thawed sperm before transfer to fertilization medium; and by co-incubating sperm and eggs in a Transwell insert. The latter two procedures suggest that a soluble factor released by damaged sperm is largely responsible for the poor post-thaw fertility of cryopreserved mouse sperm. Dilution or removal of this putative factor increases IVF success.

Recently, significant improvements to the IVF protocol have been published, including the addition of methyl-beta-cyclodextrin (MBCD) to the preincubation medium (TYH), and the addition of reduced glutathione to the fertilization medium (HTF). The former promotes sperm capacitation, while the latter appears to act by softening the zona pellucida. Preincubation in medium containing MBCD; reduction in immotile/damaged sperm or factors released by them; and the use of fertilization medium containing reduced glutathione, improve both fertilization rate and robustness of IVF procedures. Perhaps it is time to determine if the complexity of IVF protocols can be reduced, for example, by using a cryoprotectant comprised of raffinose dissolved in water, and adding MBCD to the fertilization medium.

Strain differences in the number and quality of eggs obtained following superovulation with PMSG and hCG are well known, and impact on the success of IVF. Novel superovulation procedures have been introduced to improve the number and/or quality of eggs superovulated, including injection of a GnRH agonist to improve the quality of 129 eggs, and the injection of inhibin neutralizing antibody. These procedures will be mentioned briefly.

## IVF using Cryopreserved Unfertilized Oocytes

**Naomi Nakagata**

Center for Animal Resources & Development (CARD), Kumamoto University, Japan

Since the first successful report into oocyte freezing, many papers concerning the cryopreservation of mouse oocytes have been published. However, a simple and practicable freezing method for unfertilized C57BL/6 mouse oocytes has yet to be established. C57BL/6 is the prevalent inbred strain and is used for large-scale knockout programs. This means that many researchers receive the opportunity to expand the C57BL/6 mouse population via in vitro fertilization and embryo transfer techniques for use in their studies. However, in vitro fertilization generally requires the use of a few knockout males and dozens of C57BL/6 female mice to ensure the production of a sufficient number of embryos. This forces us to keep a large number of female mice, which requires a considerable amount of space and a lot of money. Furthermore, female mice must be superovulated before oocyte collection can be carried out. However, if we are able to successfully freeze C57BL/6 oocytes, we will be able to carry out in vitro fertilization using these oocytes when needed. In this study, we carried out in vitro fertilization using three different oocyte-sperm combinations: cryopreserved oocytes and fresh sperm; cryopreserved oocytes and sperm stored at cold temperature; and cryopreserved oocytes and frozen sperm taken from a C57BL/6 mouse. Nearly all of the cryopreserved oocytes were recovered, of which around 90% were morphologically normal. Those oocytes were then used for in vitro fertilization, resulting in over 80% of oocytes being fertilized in each of the three cases.

## Practical applications of ICSI in mice

### Atsuo Ogura

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Intracytoplasmic sperm injection (ICSI, or microinsemination as a broad term) has been used widely to study the mechanisms of mammalian fertilization and to rescue male-factor infertility in humans and animals. ICSI in the mouse has been very successful since the use of a Piezo-driven micromanipulator for sperm injection into oocytes was pioneered by Kimura and Yanagimachi in 1995. The high fecundity achieved in mice using ICSI is attributable to: 1) efficient oocyte activation by oocyte activation factor(s) introduced with the injected spermatozoon, and 2) the high ability of fertilized oocytes to develop, even after harsh micromanipulation in vitro. Furthermore, recent technical advances including the development of high-resolution inverted microscopy and fine-moving micromanipulators have enabled us to identify immature gametes at each stage of spermatogenesis and to inject them into oocytes very easily. In the mouse, elongated spermatids can be used as efficient substitute gametes; if necessary, round spermatids can also be used for producing healthy offspring. Therefore, there are many practical applications of mouse ICSI, including the propagation of valuable genetic strains, production of transgenic mice, research into mutant strains, accelerated production of congenic strains, spermatogonial stem cell transplantation and germ cell production in vitro. The specialized knowledge and skills associated with ICSI will greatly help in managing murine genetic archives and in advancing research in biomedicine.

## Novel concepts in mouse production and preservation

**K. C. Kent Lloyd**

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The field of mouse biology continues to advance at breakneck pace. Today, more than at any other time in history, there are more mutant mouse models available and accessible as biomedical research surrogates for humans and animals for the study of biology and disease mechanisms. Recognition of the scientific value of spontaneous and induced mutations of the mouse genome for understanding and resolving biological questions has propelled the genetically-altered mouse as the #1 animal model for research and discovery. Over the next 10 years, multimillion dollar investments by government, commercial, and not-for-profit entities across the globe promise not only the production of mouse mutant mice expressing mutations for every gene in the mouse genome, but also broad-spectrum phenotyping data and imagery that will dramatically increase our knowledge and reveal new insight into gene function to justify exploratory, hypothesis-driven research.

But this unparalleled success in the utilization of the mouse as a research model also brings with it some sobering issues and challenges. The explosion in production of mice for research is continuing...and expanding, with no sign of contraction in the foreseeable future. In addition, the increasingly specialized expertise and dedicated technical infrastructure continues to increase costs in the face of limited resources. Further, despite recent advances in genome sequencing and knockout technology, we are still at the "young adult" stage of scientific development when it comes to the potential to fully manipulate the mouse genome. Over the span of about a generation, we have moved from single gene mutations in all tissues to the ability to induce a tissue-specific targeted trap allele to become "wildtype" before ultimately converting it into a fully deleted knockout allele. As breathtaking as these accomplishments seem, the future is equally as bright. New ideas and technologies will enable even more sophisticated allele types, polygenic mutations, libraries of humanized and other "specie-ized" mice, and more.

For technology to keep pace with the evolution of scientific sophistication in an era of austerity, we must continue to enhance and improve ways to produce and preserve mutant mice for research. We've moved beyond the "how" to produce and preserve questions (e.g., blastocyst injection) to "how fast" (e.g., morulae aggregation), "how efficient" (e.g., increasing the number of desired genotype per litter), and "how effective" (e.g., increasing the number of Cre-recombined alleles per generation) production and preservation can become. The focus on these questions will decrease the time and cost to produce and preserve high quality mutant mouse models with increased scientific relevance and reliability.

Research and investigation today in a number of areas with the potential for technological leaps tomorrow will, if successful, contribute to lower cost, faster time, and higher quality of mutant mouse models used for research. Some of these areas include reprogramming of somatic cells and gene targeting in induced pluripotent stem (iPS) cells, restoration of motility in dried sperm, laser-assisted in vitro fertilization (IVF), molecular vectors as reliably recoverable genetic storage formats, and warp-speed congenics, to name just a few. Technological advances in these and other areas will be needed to meet the challenges posed by provocative biological questions and the demand for new knowledge.



## Generation of germ line transmitting chimeras from targeted C57BL/6N ES cells by aggregation with outbred host embryos

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The Toronto Centre for Phenogenomics (TCP) opened in 2007 as a result of an innovative collaboration among Mount Sinai Hospital, The Hospital for Sick Children (SickKids), St. Michael's Hospital and the University Health Network. The TCP Transgenic (Tg) Core is an amalgamation of two facilities originally at Mount Sinai Hospital and SickKids that were in operation since the early 1990s and successfully implemented the generation of mouse embryonic stem cell (ESC) chimeras by morula aggregation and the tetraploid complementation assay. The TCP Tg Core provides such services as pronuclear microinjection, gene targeting, derivation of new ESC lines, generating chimeras using genetically modified ESC clones from Toronto ESC facilities and publicly available ESC libraries at the International Gene Trap Consortium (IGTC) and International Knockout Mouse Consortium (IKMC) repositories.

The TCP Tg Core has an ongoing collaboration with the Canadian Mouse Mutant Repository (CMMR), the repository for the Canadian contributions to the IGTC and IKMC resources. Together the TCP Tg Core and CMMR demonstrated the successful generation of germline transmitting chimeras with targeted C57BL/6N ESC clones from the IKMC and are integral partners in the Genome Canada NorCOMM2 and NIH KOMP2-DTCC projects. These two projects are part of the International Mouse Phenotyping Consortium (IMPC) that will generate and phenotype mutant mouse lines from IKMC ES cells.

Efficient methods to generate germ line transmitting (GLT) chimeras are needed to fully realize the value of IKMC gene targeted resource and to meet the ambitious goals of the IMPC – to generate and phenotype 5,000 new knockout mouse lines in 5 years. The most common approach to produce chimeric mice from C57BL/6N ESC is to inject them into albino blastocysts (e.g. BALB/c, C57BL/6J-Tyrc-2J, C57BL/6J-Tyrc-Brd) or eight-cell stage embryos (e.g. SW). We combined years of experience with outbred morula ↔ ES cell aggregation with Knockout Serum Replacement™-based medium containing inhibitors for the culture of C57BL/6 ESC to develop an efficient alternative to microinjection methods. We have produced chimeras from >100 targeted IKMC ES cell clones derived from different C57BL/6N parental ES cell lines using this method. Based on the ESC clones that completed breeding - 74% transmitted through the germline. Overall GLT rate based on all attempted clones is 57%. This includes the generation of chimeras from 34 NorCOMM clones demonstrating 88% GLT rate (<http://www.ncbi.nlm.nih.gov/pubmed/20582321>).

We will present our most recent results along with an overview of our current technology development approaches to improve GLT rates from IKMC ES cells.

## **AMMRA and AMPC: coordinated efforts for mouse resource and phenotyping in Asia**

**Xiang Gao**

Model Animal Research Center of Nanjing University, China

Following the trend of coordinated activity on mouse resource sharing and generation by IKMC, we established the Asian Mouse Mutagenesis and Resource Association with institutes from Japan, China, Korea, India, Singapore and Taiwan in 2006. This organization has helped to advocated several key mouse mutagenesis programs in China, Japan, Korea and Taiwan. It also promoted the exchange program among Asian countries. In China, two major programs were completed in the past 5 years, including the 150 mouse cKO project led by Model Animal Research Center of Nanjing University and 1000 PB insertion strain project led by Developmental Biology Institute of Fudan University. In addition, Japan, Korea and Taiwan all began to implement large scale mouse mutagenesis program in the last 3 years. We are in the process to establish the unified database portal for better resource sharing among Asian scientists.

Moreover, since RIKEN BioResource Center in Japan and Model Animal Research Center of Nanjing University in China have join the AMPC, we established the Asian Mouse Phenotyping Consortium in 2010. AMPC will promote the standardizing the mouse phenotyping protocols among the major institutes in Asian. Moreover, we hope to develop some add-on assays which may help to decipher more information for gene function.

In summary, AMMRA and AMPC will play important role to assistant the globe effort for analyzing the gene function by mouse models.

## Mouse Strain Cryopreservation in Australia

### Stuart Read

Australian PhenomeBank Curator, The Australia National University, Canberra, Australia

Since its inception in 2005 the cryopreservation and IVF techniques used by the Australian Phenome Bank (APB) have seen only minor advances, with poor recovery efficiencies for strains on C57BL/6 background continuing to hamper progress. Positive improvements to cryopreservation were made in efficiencies of processing large numbers of mice rather than the technique itself. In 2010-11 it became clear small IVF labs around Australia were each working independently to improve IVF efficiencies resulting in small improvements and slow progress. In 2012 the APB is developing plans for a more integrated Australian approach for cryopreservation and IVF technique development and training.

Testing of a modified cryopreservation and IVF technique, incorporating recommendations of Takeo and Nakagata, has resulted in dramatic improvements in IVF efficiencies. Samples from genetically modified strains that had previously failed to fertilise C57BL/6 oocytes now result in 80 – 90% fertilisation rates. Comparisons of current APB cryopreservation and IVF techniques to the published “Jax” technique and the more recently published Takeo and Nakagata technique will be discussed. The results of experiments testing variables such as sperm donor age, oocyte donor age, incubation times (both pre-cryopreservation and sperm – oocyte mix) and sources of media and plasticware will also be presented.

## The Canadian Mouse Mutant Repository (CMMR)

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The Canadian Mouse Mutant Repository is Canada's national mouse mutant repository and is the repository for Canada's International Knockout Mouse Consortium (IKMC) projects, NorCOMM and NorCOMM2. The CMMR has an ongoing collaboration with Toronto Centre for Phenogenomics (TCP) Transgenic Core and together offer fee-for-service cryopreservation of embryos and sperm, cryorecovery, re-derivation and distribution services. To support Canada's IKMC projects and academic users, the CMMR uses current best practices for cryopreservation. In particular, the state-of-the art with respect to sperm cryopreservation is rapidly changing. Since fall 2009 we have been cryopreserving sperm using cryoprotectant with monothioglycerol (MTG) [1], though with some modifications from published protocols based on work previously done at CMMR [1,2]. Recent publications have outlined novel cryoprotectant and modifications of in vitro fertilization (IVF) protocols that significantly improve post-thaw fertilization rates [3-5]. We have begun testing this modified IVF method, based on the protocols implemented by EMMA, using sperm we previously cryopreserved with cryoprotectant with MTG. Our data show that inclusion of methyl- $\beta$  cyclodextrin in pre-incubation medium and reduced glutathione in high calcium HTF medium improves post-thaw fertilization rates compared to the methods we previously used. We will present data from sperm cryopreserved with MTG and used for post-thaw IVF in two different methods. Our goal is to improve cryorecovery rates for our previously frozen sperm and to continue to freeze new strains using state-of-the-art methods.

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## Cryopreservation Technology Status in South America

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Cryopreservation of semen and embryos are widely used in our region in farm species since several decades ago. Nevertheless, this technology is far from being applied in large scale in biomedicine in animal houses that breeds and maintains transgenic and KO murine lines. One of the major causes may be the lack of expertise in technical personnel or the costs of the equipments required for embryo freezing. In 2006, the recently launched Institut Pasteur in Montevideo opens a transgenic core facility that includes the cryopreservation of murine sperm and embryos service. It was the first facility in Uruguay and one of the few in South America (one in Chile; five in Brazil). Since then we have cryopreserved about 20 transgenic lines from researchers of Uruguay and neighboring countries, with a growing tendency of laboratories to preserve valuable lines. For sperm freezing, the Jackson Laboratory protocol (2011) is currently used with acceptable results. The method of choice for 8-cell embryos was slow freezing at the beginning, using the protocol of Renard & Babinet (1984). In 2010 the laboratory started to offer the vitrification method, using a modified Vitrification Spatula protocol (Tsang & Chow, 2009). We compared the performance of these two methods in the cryopreservation of a transgenic line in terms of recovery, survival and blastocyst development rates. With this method, we obtained a higher recovery rate (96.4% vs. 84.9%), higher post-thawing survival rate (99.3% vs. 84.4%) and higher development rate (92.5% vs. 52.6%) than with slow freezing method ( $P < 0.05$ ). Development rate for control group, which have not suffered any cryopreservation/thawing-warming process, was 94.3%. In our hands, vitrification of 8-cell in vivo obtained embryos promises to be a reliable alternative to the slow-freezing method used in our laboratory, and a simpler and more economic method to be applied in any core facility having the necessary expertise.

In that sense, we consider the archive of mouse mutant lines as important as its generation, in order to preserve all the effort made to obtain these transgenic or KO lines. Regional efforts have been made with the aim to spread the cryopreservation technology in Brazil, Argentina, Chile and Uruguay, bringing researchers from reference centers of United States and Europe to regional courses. Recently, Brazil started the initiative to create a national repository of germplasm, with the support of experts working in EMMA, NIH, Institut Pasteur in Paris and Institut Pasteur de Montevideo. The aim of this initiative is to generate a similar network to EMMA repository for our region, including regional centers as Institut Pasteur de Montevideo.

In conclusion, in spite there are some laboratories in our region that performs embryo and sperm cryopreservation following international standards and offers this technology to researchers, there is still a long way to go in order to have more centers and trained technicians throughout South America. In that sense, regional courses of excellence and changing the researcher's attitude towards this technology is our current challenge for the next years.

## United States of America: Mutant Mouse Regional Resource Center (MMRRC)

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The Mutant Mouse Regional Resource Center (MMRRC) system is a National Institutes of Health (NIH)-funded biorepository system dedicated to the archiving, preservation, protection, and distribution of genetically-altered mutant mouse models in support of biological and biomedical research. There are 4 MMRRC's in the MMRRC National Consortium, regionally distributed at academic and private institutions across the United States: the MMRRC at University of North Carolina-Chapel Hill, the MMRRC at the University of Missouri-Columbia, the MMRRC at the Jackson Laboratory Maine, and the MMRRC at the University of California Davis. In addition, an Informatics, Coordination, and Service Center (ICSC) provides informatics, technical, and customer service both to the 4 regional MMRRC's and to the biomedical community of research scientists who use the MMRRC. The regional MMRRC's accept mice from research scientists and conducting quality control procedures to ensure their identify and viability. Mutant mouse lines accepted into the MMRRC are unique, scientifically meritorious, usually published, and do not exist at any other repository or archive. Afterwards, the MMRRC's create a cryopreserved germplasm and/or embryonic stem (ES) cell archive from the mouse lines maintain them for distribution. Mouse lines that are in higher demand are maintained as live breeding animals for ready availability. These and all others mouse lines are also available in a variety of cryopreserved formats for either distribution or for recovery to live mice and distribution. Approximately 35,000 mutant alleles are currently archived and available for distribution as live mice, cryopreserved germplasm, and/or ES cells from the MMRRC. The MMRRC's also conduct resource-related research in order to improve and enhance the operations of the MMRRC National Consortium, including in the area cryobiology, cryopreservation, cryorecovery, and artificial reproductive techniques.

## SOME USEFUL WEB LINKS

AMMRA: <http://www.ammra.info/>

AMPC: <https://database.riken.jp/sw/en/Homepage/cria324s1i/>

APB:

<http://pb.apf.edu.au/phenbank/foreignPageImport.html?page=http://pbstatic.apf.edu.au:80/phenbank/home.htm>

APN: <http://www.australianphenomics.org.au/>

CARD: <http://card.medic.kumamoto-u.ac.jp/card/english/index.html>

CMMR: <http://www.cmmr.ca/>

CNB: <http://www.cnb.csic.es/~criocnb>

CNR-EMMA: <http://www.emma.cnr.it/>

CSIC: <http://www.csic.es>

EMMA: <http://www.emmanet.org>

EUCOMM: <http://www.knockoutmouse.org/about/eucomm>

GMC: <http://www.mouseclinic.de/>

RIKEN: <http://www.riken.go.jp/engn/r-world/research/lab/brc/index.html>

ICS: <http://www.ics-mci.fr/>

IKMC: <http://www.knockoutmouse.org/>

IKMP: <http://www.mousephenotype.org/>

INFRAFRONTIER: <http://www.infrafrontier.eu>

IPM: <http://www.pasteur.edu.uy/>

ISTT: <http://www.transtechsociety.org>

JAX: <http://www.jax.org>

KOMP: <https://www.komp.org/>

MGU-MRC: <http://www.har.mrc.ac.uk/>

MMRRC: <http://www.mmrrc.org/>

NorCOMM: <http://www.norcomm.org/>

TCP: <http://www.phenogenomics.ca/>

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## (11th) Transgenic Technology (TT) Meeting: TT2013

**Baiyun International Convention Center**

**Guangzhou/Canton, P.R. China, 25-27 February 2013**

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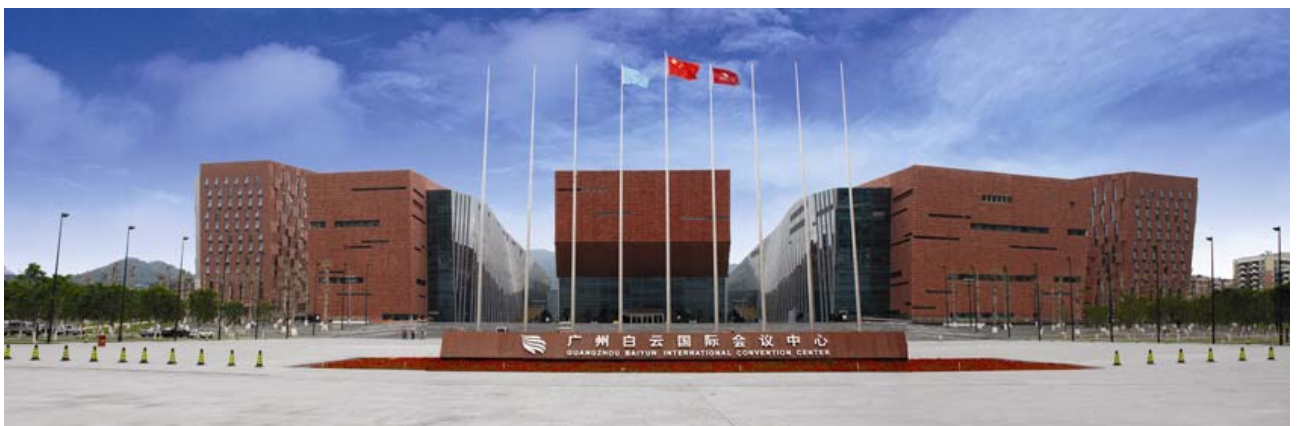
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**International Society for Transgenic Technologies**

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The International Society for Transgenic Technologies (ISTT) was founded in January 2006. The Society seeks to foster communication and technology sharing, to enhance scientific research, to advance the field of animal transgenesis, particularly as it applies to useful experimental models in biology, medicine and biotechnology, and to represent the interests of scientists, technicians, and graduate students working in the field of transgenic technologies. The ISTT sponsors a series of periodic international meetings known as the Transgenic Technology (TT) Meetings.

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