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Cryopreservation of Aquatic Biomedical Models Workshop

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Workshop Report

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Executive Summary

The National Institutes of Health's (NIH) Office of Research Infrastructure Programs (ORIP) hosted the Cryopreservation of Aquatic Biomedical Models Workshop on January 7, 2017, in conjunction with the Eighth Aquatic Animal Models of Human Disease Conference in Birmingham, Alabama; the agenda is included as Appendix A. Approximately 25 experts in the field of cryopreservation of aquatic models, representing the cryopreservation scientific community at large, attended the workshop; a participants list is provided in Appendix B. The goal of the workshop was to assess the status of germplasm cryopreservation in various aquatic models to allow the scientific representatives of the at-large community to come to a consensus regarding specific, actionable recommendations for NIH ORIP to consider.

The workshop included a session devoted to opportunities for the cryopreservation of aquatic species, in which presenters provided information about current efforts and approaches to preservation of aquatic model germplasm. Another session focused on current efforts addressing reproducibility and standardization for repository development and throughput/scalability technologies. During the third session, resource center directors provided their perspectives on their centers' current cryopreservation status and barriers. In the final session, the participants developed recommendations to ORIP regarding aquatic model reproductive and cryopreservation research. Their recommendations are to:

- Establish a comprehensive, centralized unit ("hub") to programmatically develop training for and documentation of cryopreservation of aquatic model systems. This will include the development of species-specific protocols and approaches, outreach programs, community development and standardization, a freezing service, and the training of the next generation of aquatic cryopreservation experts.

- Provide mechanisms to support innovative technical advancements that will increase the reliability, reproducibility, simplicity, throughput, and efficiency of the cryopreservation process, including vitrification and pipelines for embryos, eggs, sperm, oocytes, stem cells, and somatic cells of all aquatic species. This recommendation encompasses basic cryopreservation knowledge and engineering technology, such as microfluidics and processing technologies.

- Implement mechanisms that allow stock centers to increase their planning, personnel, and ability to secure genetic resources and develop their own repositories as well as allow them to interact within an integrated, comprehensive repository network for aquatic model species. The network may be hosted by the hub so standards can be maintained.

Workshop Report

Background

During the past century, aquatic animal species have demonstrated their utility as powerful models for studying human development, behavior, genetics, and disease. Zebrafish, medaka, *Xiphophorus*, and *Xenopus* are increasingly valuable to biomedical researchers because they provide critical clues to the biological mechanisms that underlie human health and disease. The ability to produce transgenic, knockout, and mutant lines of many aquatic species has provided biomedical researchers with a variety of models for the study of human diseases. Despite the significant cost to generate these lines, reliable and cost-effective approaches for long-term preservation of these scientific resources still are lacking. Although cryopreservation of sperm is the sole and proven method for the long-term maintenance in many aquatic models, no other approaches—such as additional germplasm formats (oocyte, embryo, ovarian tissue, testicular tissue, or embryonic stem cells) or reproductive engineering technologies—currently are available to aquatic researchers.

To address this gap, the National Institute of Health's (NIH) Office of Research Infrastructure Programs (ORIP) sponsored the Cryopreservation of Aquatic Biomedical Models Workshop in conjunction with the Eighth Aquatic Animal Models of Human Disease Conference, held January 7–12, 2017, in Birmingham, Alabama. The objectives of the workshop, held on January 7, 2017, were to (1) assess the status of germplasm cryopreservation in various aquatic models; (2) identify the obstacles, opportunities, and priorities that may address the need for improved methods; and (3) evaluate novel and emerging research and technologies that might lead to the successful preservation of other germplasm formats.

Summary of Presentations and Discussions

Introduction and Welcome

Mary Hagedorn, Smithsonian and Conservation Biology Institute and Hawaii Institute of Marine Biology, Kaneohe, Hawaii; and Miguel Contreras, ORIP, NIH, Bethesda, Maryland

Dr. Mary Hagedorn welcomed the participants and provided a cautionary tale about securing genetic resources. Coral is the most restricted reproductive species on the planet, reproducing only 2 nights per year. More than 400 species of coral exist in the Great Barrier Reef, but only 11 species have been banked, and until recently the Great Barrier Reef Marine Park Authority (which has supervisory oversight) has been uninterested in cryopreservation. Given the recent trends in coral bleaching, with more frequent and larger die-offs, species preservation is particularly critical. All opportunities to preserve aquatic resources must be taken, and cryopreservation banks can facilitate these efforts. The assembled experts can help to secure important resources, which then can be used as models for wild populations and food resources. Given that the NIH is open and willing to support the preservation of genetic diversity of many types of aquatic species, this is an amazing opportunity that should be taken up with seriousness.

Dr. Miguel Contreras acknowledged the efforts of the workshop organizing committee and provided an overview of ORIP, which was established in 2011 when the National Center for Research Resources was dissolved and several of its programs reassigned to the Office of the Director. ORIP supports the NIH's mission by providing resources to drive research discoveries; the office is interested in all species that can provide understanding of human health and disease in a broad context. ORIP developed this workshop to provide an overview of the current cryopreservation field, identify barriers that need to be addressed to move the field forward, learn about new opportunities and available technologies, and obtain actionable recommendations so that the office can determine where to best place its resources.

Session 1: Opportunities for Cryopreservation of Aquatic Species: Eggs, Embryos, Reproductive Tissues, and Methods

Session Chair: Stuart Meyers, University of California at Davis, Davis, California

Development of a Universal Platform for Aquatic Germplasm Cryopreservation
John Bischof, University of Minnesota, Minneapolis, Minnesota, and Mary Hagedorn, Smithsonian and Conservation Biology Institute and Hawaii Institute of Marine Biology, Kaneohe, Hawaii

Successful cryopreservation of lower vertebrate embryos, including zebrafish, has remained an elusive goal for the past several decades. Multiple barriers exist for the cryopreservation of zebrafish embryos, including their large size, the presence of multiple internal compartments, low membrane permeability to cryoprotectants, and chill sensitivity. Microinjection of cryoprotectants and rapid cooling were used to overcome the permeability barrier and chill sensitivity. The relatively massive size of the embryo, however, encourages the formation of lethal intracellular ice on warming, even under the most rapid convective conditions. This has necessitated a search for new ways to rapidly and uniformly warm these systems.

In this study, laser irradiation of gold nanorods (GNRs) was used to warm zebrafish embryos that had been previously cooled and stored in liquid nitrogen. Specifically, the researchers developed protocols for micro-injecting a cryoprotectant (propylene glycol [PEG]) and GNRs into zebrafish embryos followed by rapid and uniform cooling at roughly 90,000°C/minute. Subsequent ultra-rapid warming at rates of 1.3×10^7°C/minute are estimated from a 1,064-nm laser pulse (approximately 1 millisecond) that heats the GNRs and surrounding embryo, thereby outrunning any intracellular ice formation. The results from 11 trials ($n = 203$) demonstrated viable embryos with consistent structure at 1 hour (31%), continued development at 3 hours (16%), and continued development and movement at 24 hours (5%) after warming versus 0% at all time points in convectively warmed controls ($p < 0.001$, ANOVA). This ultrafast laser warming technology has the potential to transform banking of fish systems while also establishing a platform for conserving the germplasm of other vertebrate and nonvertebrate egg and embryo systems.

The researchers have concluded that the zebrafish embryo can be cooled successfully with the Cryotop® Method, embryo storage is possible in liquid nitrogen, zebrafish embryos can be rewarmed by a 1,064-nm (1 millisecond) laser pulse, and the approach is physically scalable for germplasm as large as 1.4 mm. Further experimentation and optimization is planned.

Discussion

A participant asked whether conductivity changes with the GNRs. Dr. John Bischof indicated that it does not change.

The participants discussed the use of dimethyl sulfoxide (DMSO) versus PEG, noting that although DMSO may be able to enter the cells of organisms that are less permeable, the yolk of zebrafish and perhaps many other species may be impermeable to DMSO.

Because a uniform distribution of GNRs is necessary, the researchers are in the process of demonstrating uniformity through optical microscopy. The GNRs do not need to be in every cell, just sufficiently, uniformly distributed.

A participant asked whether the method would work in other fish species. Dr. Bischof thought that it would.

Production of Viable Trout Offspring Derived from Frozen Testis Via Germ Cell Transplantation
Goro Yoshizaki, Tokyo University of Marine Science and Technology, Tokyo, Japan

Many salmonid species recently have become at risk of extinction. For fish species whose eggs cannot be cryopreserved, establishment of techniques to preserve genetic resources other than egg and embryo cryopreservation is imperative. In the present study, spermatogonia from male trout were transplanted into the peritoneal cavity of newly hatched sterile triploid salmon. Transplanted trout spermatogonia colonized the gonads of sterile salmon recipients. In male recipients, transplanted spermatogonia underwent spermatogenesis. Further, in female recipients, transplanted spermatogonia underwent oogenesis. At 2 to 3 years following transplantation, triploid salmon recipients produced only donor-derived trout sperm and eggs. By artificial insemination with the sperm and eggs obtained from the triploid salmon recipients, only donor-derived trout offspring were produced. Combined with cryopreservation of spermatogonia, the present technique makes it possible to preserve fish genetic resources and to revive extinct species when necessary. The researchers recently confirmed that this technology also is applicable to zebrafish and medaka.

Discussion

A participant asked about the effects of evolution. Dr. Goro Yoshizaki explained that if the fish belongs to the same genus, the success rate is high. When the fish belongs to different genera, the combination is important; inducing egg generation is more difficult than sperm generation.

A participant asked about the size of the testes in the samples. Dr. Yoshizaki indicated that, although it depended on the maturity, the approximate length was 3 to 5 cm, and the diameter was approximately 2 mm.

A participant asked about the rate of freezing. Dr. Yoshizaki stated that the sample was frozen at 1°C/minute and then maintained at -80°C for 90 minutes, followed by transfer to and immersion in liquid nitrogen.

A participant asked whether the researchers had tried to dissociate the cells first. Dr. Yoshizaki explained that the laboratory had and it works, but the focus is on the protection of endangered species, so whole testis freezing is much easier. The survival rates between dissociated cells and whole testis are similar.

A participant asked about germ cell purification. Dr. Yoshizaki indicated that when whole testis or ovarian cells were transplanted, germ cell purification was not necessary. Stem cells can migrate to the recipient.

Lyopreservation of Sperm
Sankha Bhowmick, University of Massachusetts, Dartmouth, Massachusetts

The need for simple preservation technologies is becoming increasingly urgent as biomedical science and biotechnology develop a complex array of cellular- and tissue-based products. Desiccation preservation of nucleated mammalian cells offers an attractive alternative to liquid nitrogen cryopreservation protocols by potentially allowing ambient temperature storage. The inspiration comes from nature, where anhydrobiotic organisms survive extreme drought by moving to metabolic stasis, followed by resumption of life when water becomes available. Expressing intracellular nonreducing saccharides like trehalose seems to be the adaptive pathway taken by most of these organisms. A similar strategy has been adopted for desiccation preservation of mammalian cells; however, sophisticated techniques are required for delivering intracellular disaccharides. During the past decade, the researchers have focused on murine and bovine sperm as important models for desiccation preservation. Murine sperm preservation techniques require drastic new strategies because of an exponentially increasing pool of transgenic mice. In this

research, desiccation preservation strategies focused on recovering intact DNA that was used to fertilize eggs through intracytoplasmic sperm injection. The use of trehalose and a calcium chelator (EGTA) has allowed successful long-term preservation of murine sperm DNA. For bull sperm, the requirement was more stringent where motility recovery was essential for the dairy industry. Using combinatorial methods of intracellular trehalose along with iron chelators (e.g., desferel) and osmolytes (e.g., sorbitol), desiccation tolerance has been improved in bovine sperm. The results indicate that improving osmotolerance is a first important step in moving toward desiccation tolerance in mammalian cells. Further studies elucidating the dynamic transport events that lead to cellular desiccation stresses are underway.

Practical Applications of Somatic Cell Nuclear Transfer (SCNT) in Zebrafish
Jose Cibelli, Michigan State University, East Lansing, Michigan

SCNT helps to provide an understanding of the molecular mechanism of cellular reprogramming and allows germplasm conservation. To implement this protocol, activated oocytes were used and the method verified. Whereas use of the laser is relatively easy, moving through the micropyle (i.e., introducing cells in the same location sperm must enter) is more challenging. Ultimately, however, the method produced a perfect match with the donor cell with no contamination from the recipient egg. Because donor cell type is important in mammalian cloning, the researchers examined whether this is the case in zebrafish by cloning several cell types; the highest efficiency came from mesoderm cells. The laboratory also explored freezing protocols for germplasm preservation, determining that Matrigel® and Geltrex® growth substrates, DNAC culture medium and cryoprotectant, insulin-like growth factor (IGF), a density of 60,000 cells, and addition of a ROCK inhibitor to be optimal for successful cryopreservation and thawing. Cell cycle also is important for nuclear transfer; 90% of the cells were in the Gap 0 or Gap 1 stage of the cycle. Ultimately, the researchers concluded that Tübingen/AB and Casper Fish™ can be generated by SCNT using freshly isolated and frozen-thawed cells and that primary fibroblast-like cells can be reliably derived and cultured using the approach described above. The next step is to examine improvement in oocyte activation and large-throughput compound screening using a device developed in collaboration with chemical engineers at Michigan State University.

Discussion

A participant asked about the method for dissociating the embryo for culture. Dr. Jose Cibelli indicated that the protocol involved homogenous mixing and pipetting up and down.

A participant asked whether the mitochondria are derived from the eggs. Dr. Cibelli explained that this is the case. Dr. Hagedorn asked about the best kinds of eggs. Dr. Cibelli responded that the best eggs are those from the F1 cross from Tubingen and AB lines.

Practical Method for Cryopreservation of Medaka Sperm and Its Application to Other Aquatic Models
Kiyoshi Naruse, National Institute for Basic Biology, Aichi, Japan

The researchers have established a practical method of sperm cryopreservation in medaka. This method is based on a combination of results from two published papers (Aoki K, Okamoto M, Tatsumi K, Ishikawa Y. *Zoolog Sci.* 1997 Apr;14(4):641–644; Krone A, Wittbrodt J. *The Fish Biology Journal Medaka.* 1997 9:47–48). The researchers found that 10% dimethylformamide-fetal calf serum is an efficient cryopreservation medium. This medium efficiently prevents the initiation of sperm movement and then increases viability of cryopreserved sperm after thawing. Use of glass capillary tubes allows multiple *in vitro* fertilizations from a single male; approximately 100 eggs can be fertilized with sperm from a single capillary tube. A 15-mL falcon tube embedded in crushed dry-ice is good insulator to decrease the freezing rate to induce a slow freezing method. An inexpensive and convenient method for sperm quality

control and quantification is the use of a phase contrast microscope mp4 movie of sperm followed by data processing using Adobe Photoshop and ImageJ.

Discussion

A participant asked whether the method works for *Xenopus*. Dr. Kiyoshi Naruse explained that sperm quality is the most important factor in determining whether the method will work. The trigger for activating medaka sperm currently is unknown.

In terms of the freezing rate, the researchers quickly cool the glass capillary tube from room temperature to 4°C using ice.

A participant asked whether the laboratory has determined a quantified cutoff for what is useable. Dr. Naruse explained that the researchers examine the concentration visually using video.

Panel Discussion

A participant noted that freezing a zebrafish embryo is essentially freezing the chorion and the yolk sac. Some of the frozen yolk sacs can be thawed and survive to 24 hours. The cells are not viable after 24 hours for many reasons (e.g., chorion hardening, oxygen issues). Drs. Hagedorn and Bischof have only early results from the study, and many opportunities exist to explore this area. A participant noted that uniformity is important, and once the current questions have been answered, it will be possible to focus on the specific biological issues and discuss the different reasons some embryos die and others live. Researchers are on the verge of obtaining an embryo survival rate of 50%, but barriers to survival must be identified and addressed.

A participant noted that freezing is not the bottleneck; the bottleneck is moving from 2% to 3% efficiency to 20% to 30% efficiency. Automation may not have an advantage, as a good technician can process many samples. Also, a good gene bank must have the ability to utilize many different approaches (e.g., cryopreservation, lyopreservation) and types of cells.

Once researchers determine whether and what genes are activated in the freezing and thawing process, one approach could be to co-inject GNRs with mRNA from the identified genes to suppress the apoptotic pathway. Researchers must ask the right questions so that they are able to coax the embryos to adulthood.

Session 2: Reproducibility and Standardization for Repository Development and Throughput/Scalability Technologies

Session Chair: John Bischof, University of Minnesota, Minneapolis, Minnesota

Reproducibility, Quality Control, and Standardization
Terrence Tiersch, Louisiana State University (LSU) Agricultural Center, Baton Rouge, Louisiana

Laboratories around the world have produced tens of thousands of mutant and transgenic zebrafish lines. Within the past decade, sperm cryopreservation has steadily improved to accommodate these lines, and cryopreservation is becoming routinely applied for repository development. Despite this success, cryopreservation remains problematic for most aquatic models, and results are characterized by a pronounced lack of reproducibility and standardization. Basic factors necessary for reproducibility (e.g., measuring and adjusting sperm concentration) are not employed, and large uncontrolled variation is an intrinsic condition that greatly reduces overall success and efficiency. In addition, the primary quality control assay (motility) is either not utilized or used insufficiently; when this assay is used for initial characterization of samples, it is not always predictive of post-thaw performance. These problems exist

because current methods for assaying necessary factors, such as concentration and motility, are viewed as unnecessary, cumbersome, time consuming, and difficult to perform; when performed, they are plagued with the problems of lack of standardization.

Continuation of the current practices for freezing and sample sharing includes at least the following three levels of problematic consequences:

1. Small laboratories do not typically have access to reproducible technology, and in-house backup of lines could fail entirely or be difficult to perform. This lack of reproducibility creates doubt and requires effort and cost to freeze additional samples or might require regeneration of lost lines, increasing the effort and cost for research. In addition, laboratories may have to submit lines several times to resource centers with the accompanying recordkeeping.

2. Resource centers and repositories experience the consequences of poor reproducibility. Because of a lack of standardization, resource centers must accommodate a wide diversity of sample containers and labeling, which causes problems for physical storage and recovery. They also must deal with multiple idiosyncratic protocols, with extra effort required to discern thawing and fertilization conditions. Additionally, when samples and lines have to be submitted repeatedly, which requires multiple thawing and fertilization attempts, work load and costs increase. This can lead to thawing of all available samples for a line to ensure fertilization, thus wasting samples and leading to the risk of losing lines that were considered to be secure. Overall, this can impair the reputation of the resource center to protect or recover lines, despite the fact that the sample was derived from a submitting laboratory. This may prompt groups to generate mutants without utilizing resource centers, ultimately slowing research and weakening the connection of the centers with their research communities. To address this, the resource center must receive animals and perform the freezing for laboratories that cannot.

3. Users of lines from resource centers also experience consequences. They depend on timely reconstitution of frozen lines, and if there are problems in regenerating frozen material and availability of these lines is delayed, the recipient laboratories lose valuable time. Alternatively, if the line is not recovered, they are charged a recovery (thawing) fee for material that they did not receive. This also erodes trust in the resource center, and the laboratory may have to generate particular mutants, further stalling projects and resulting in negative effects on performance of funded research.

These problems constitute an enormous unnecessary inefficiency, especially if multiplied across the numbers of males (and females) needed to back up tens of thousands of research lines that require freezing for maintenance. With the inclusion of relevant quality control steps (such as cell concentration and motility assessment), however, the likelihood of failure for properly handled samples decreases, and ultimately, the variability in use of thawed sperm could be largely confined to female conditioning and egg quality.

Discussion

A participant asked whether Dr. Terrence Tiersch stays current with the thinking of such groups as the National Association of Animal Breeders and the biobanking community. Dr. Tiersch indicated that he does stay current with these types of groups; Dr. Harvey Blackburn of the U.S. Department of Agriculture (USDA) is another good resource. Dr. Tiersch also noted that the private sector has devoted resources to rapid genetic improvement of bovines. Although the federal government also is involved, the private sector drives innovation. A participant noted that interagency collaboration is needed to explore innovations for species other than bovines. Communication is important because organizations and institutions have different interests and languages. Dr. Tiersch agreed with the importance of including

different institutions and organizations and commented that not performing quality control only shifts the cost to later in the process (i.e., thawing and use). Each of the hundreds of species in sectors devoted to wild fish, endangered species, imperiled organisms, biomedical models, and agriculture has its own community; it would be beneficial for these communities to collaborate. It also is necessary to develop other mechanisms so that different goals and scales can be addressed comprehensively.

Microfabricated Devices for Standardization, Reproducibility, and Throughput of Sperm Cryopreservation
Todd Monroe, LSU, Baton Rouge, Louisiana

Central repositories are refining protocols of cryopreservation to maintain and protect the genetic resources of thousands of lines of aquatic model species. This process is hindered, however, in individual research laboratories where freezing and analysis of gametes is problematic because of the lack of reproducibility and standardization. For example, sperm motility assessment lacks standardization across and within laboratories because manual methods are subject to human variation. Computer-assisted sperm analysis (CASA) has improved reproducibility over manual estimation but still lacks control over small sample volumes and short motility lifetimes in many aquatic species.

These challenges can be addressed with microfluidic platforms in microfabricated systems that have been used to shorten analysis times, reduce volumes of reagents, and enable new discoveries in cell biology. These microfluidic "lab-on-a-chip" devices have been utilized in studies of gamete and embryo physiology, where microchannels have been used for reliable delivery of sperm to oocytes, gamete and embryo isolation and culture, and sperm sorting and separation. Achieving sperm activation in freshwater species for CASA requires mixing of the sperm sample with water, which is difficult at the microscale because of the very low Reynold's number, where laminar fluid flow streams will not mix unless unique microchannel geometries are utilized. These micromixers have found promise in activating zebrafish sperm more rapidly and reproducibly than has been accomplished by manual mixing. The use of computational fluid dynamics, computer-aided design, and soft lithography technologies enables the simulation, design, and fabrication of microfabricated lab-on-a-chip devices to improve consistency in the handling and analysis of small samples.

The researchers envision the use of microfluidic devices for the activation of aquatic sperm cells overcoming several hurdles in sperm cell activation studies. These have the potential to improve cryopreservation protocols significantly by allowing high-throughput testing of different cryopreserved samples under highly controlled conditions. This work also serves as a potential for much-needed standardization; both the microfluidics and numerical methods used are robust and can be used to reduce the variability within and across aquatic sperm research laboratories and repositories.

Discussion

Dr. Hagedorn asked whether the chips can be reused. Dr. Todd Monroe explained that a silanization process halts cell adhesion, making it possible to reuse the chips. Dr. Cibelli asked about the cost. Dr. Monroe responded that the most expensive part of producing a chip is making the master transparency, which costs approximately $30. It is possible that the cost eventually could be reduced to a nickel or dime per chip.

Can Microfluidic Platforms for Droplets and Bubbles Enable High-Throughput Cryopreservation?
Cari Dutcher, University of Minnesota, Minneapolis, Minnesota

Microfluidic flow is fluid flow in a geometry with a characteristic length scale less than 300 μm. Microfluidics can be applied to biomedical and manufacturing processes and have increased convenience because of small size, lower cost, and multiple processes. Polydimethylsiloxane microfluidic devices,

which take approximately one-half day to fabricate, can be used for up to a year, and fluid flow can be driven by pressure gradients or electric fields. Droplet microfluidic experiments can allow noncontact, high throughput with "on-the-fly" changes to chemical composition and thermal conditions. Droplet deformation can be used to measure such properties as viscosity and surface tension (as in Metcalf AR, Boyer HC, Dutcher CS. *Environ Sci Technol.* 2016 Feb 2;50(3):1251-9).

Dr. Cari Dutcher highlighted examples of the biomedical applications of microfluidics from other groups. In the first example (Mazutis L, Gilbert J, Ung WL, Weitz DA, Griffiths AD, Heyman JA. *Nat Protoc.* 2013 May;8(5):870-91), droplets are used to encapsulate mouse cells along with a fluorescent probe. Antibodies can bind to the probe, releasing a fluorescent signal. Next, droplet sorting is performed with dielectrophoresis. In the second example (Brouzes E, Medkova M, Savenelli N, Marran D, Twardowski M, Hutchison JB, Rothberg JM, Link DR, Perrimon N, Samuels ML. *Proc Natl Acad Sci USA.* 2009 Aug 25;106(34):14195-200), microfluidics are used for single-cell high-throughput screening. Two-phase droplet microfluidics also may have a role in addressing the current challenge of cryopreservation, which is finding a method to rapidly transport the embryos to and from the Cryotop®. In conclusion, microfluidics provide a monodisperse, high-throughput droplet method for germplasm manipulation and may assist in several possible cryopreservation steps (e.g., cryoprotectant addition, cooling, storage, connection to warming). A possible platform for microfluidics is the potential to link, improve, or replace steps in the cryopreservation process.

Discussion

The participants discussed the logistics of using microfluidics for cryopreservation. The current platform can attain dry ice temperatures, so the next challenge to address will be attaining liquid nitrogen temperatures. Dr. Dutcher envisions the use of electrokinetic force rather than pressure in addressing the issue. The researchers currently are trying to induce the phase change, which happens at different temperatures.

Dr. Dutcher is unaware whether any laboratory has shown that a biological unit (e.g., embryo) has survived the microfluidic process, but she noted that the process is gentle. Many different microfluidic systems exist, and researchers are beginning to examine these types of questions. Microfluidics may help to address obtaining the fastest possible freezing rate and may be better than use of the Cryotop® alone.

A participant thought that examining the thermal mass of tiny droplets was a modeling exercise and was curious about the rate of heat loss. Another participant stated that the process is similar to flow cytometry, noting the difficulty in finding an aperture that will fit a zebrafish embryo. Developing microfluidics to sort fluorescently labeled transgenic zebrafish, medaka, or other eggs would be a tremendous benefit to allow high-throughput analysis of breeding experiments. The participants agreed that combining microfluidic technology with other technologies (e.g., laser) would be beneficial.

Genetic Resource Preservation and Management at the Zebrafish International Resource Center (ZIRC)

Zoltan Varga, University of Oregon, Eugene, Oregon

The ZIRC serves as the central genetic repository for wild-type, mutant, and transgenic zebrafish lines. In the past 16 years, the ZIRC has imported more than 36,000 alleles in more than 10,000 fish lines. Of these, 33,500 have been imported since 2012. To effectively manage these resources, the ZIRC improved its outdated cryopreservation method; rewrote large parts of its inventory database; and implemented flexible strategies for the import, maintenance, rederivation, amplification, and distribution of its resources. The new and improved cryopreservation protocol plays a pivotal role in all of these processes. The protocol is flexible and scalable and can be adapted for small, intermediate, and large laboratory operations, as well as in resource centers. In addition, it provides a basis for cryopreservation standards

for the zebrafish research community. The collaborative approach, which the ZIRC engaged in and established with NIH ORIP support, may serve as a model to develop cryopreservation methods for other aquatic resource centers.

Discussion

Dr. Zoltan Varga explained that the European resource centers do not use the ZIRC protocol because they believe that their current protocol works for them; Dr. Varga, however, disagrees with that belief. The center is working on publishing its protocol.

A participant asked about egg characteristics that potentially decrease the success rate of *in vitro* fertilization. Dr. Varga noted that animal husbandry and the condition of the male and female are critical. Unfortunately, it is not possible to assess the egg condition until after fertilization occurs. The assumption is that fertility rates are more dependent on egg quality than sperm quality.

A participant asked about the number of full-time equivalents and the amount of funding the center devotes to cryopreservation. Dr. Varga explained that, on a weekly basis, approximately 75% of ZIRC's efforts focus on thawing and shipping; the remaining 25% is focused on breeding and shipping. The ZIRC has not calculated the cost to freeze and maintain a line because of the difficulties in performing such a calculation. Popular lines may be requested five to six times per year, whereas other lines may be requested only once every other year.

A participant asked how many lines may have been generated but have not been submitted to the ZIRC. Dr. Varga noted that it is challenging to determine this because many lines may not be submitted because once they are, they are in the public domain. Based on the database, his educated guess would be that 15,000 to 20,000 more lines exist than currently housed at the ZIRC. The center can teach laboratories that do not want to submit their lines how to cryopreserve them.

A participant noted that cryopreservation versus long-term maintenance of live animals is extremely cost-effective and suggested that the ZIRC sell cryopreserved sperm. Harmonization of the various techniques into a platform would be an extremely powerful tool to bring the various groups together as long as the results are linked to quality control. The ZIRC has developed a powerful protocol that can be used as a model to help other communities. Transport of cryopreserved sperm is as challenging as shipping live animals. Cryogenic transport makes sense with bulk shipments, and the ZIRC used frozen sperm shipments to import large numbers of samples from large-scale genetic screens. Shipping a few lines to laboratories probably is more efficient with live animals, however.

Session 3: Perspectives from Resource Centers on Current Cryopreservation Status and Roadblocks

Session Chair: Terrence Tiersch, LSU Agricultural Center, Baton Rouge, Louisiana (substituting for Dr. Blackburn)

Dr. Tiersch described the three questions that resource center directors were asked to focus on during their presentations:

- What are the realities of using cryopreservation at the center level?
- What is needed to increase the dependability of cryopreserved germplasm?
- Why is increased success of cryopreserved gametes important?

Implementing Cryopreservation in Xenopus for the National Xenopus Resource (NXR)
Marko Horb, Marine Biological Laboratory, Woods Hole, Massachusetts

Cryopreservation in *Xenopus* is not standard, and only in the last 10 years have three published methods been tested in the genus. Few laboratories utilize cryopreservation, with most maintaining live animals. With larger numbers of *Xenopus* lines being produced, NXR must improve cryopreservation for their storage and to increase the use of lines by researchers. Even with current methods, however, frozen sperm preparations are inconsistent, and NXR frequently receives frozen sperm that produce no viable embryos. NXR is working closely with the European *Xenopus* Resource Centre to improve the methods and address these issues. When the methods were tested in *Xenopus laevis* and *Xenopus tropicalis*, a large variation was observed, and efficiency was found to be dependent on which female was used. The efficiency, however, has improved over time.

Dr. Marko Horb reiterated the need to improve the current inconsistent cryopreservation method in *Xenopus* because of the increases in transgenic and mutant frog lines being produced. Inconsistency also is increased as a result of rapid staff turnover, limited number of staff, and inexperienced staff. Compounding the issue is the reduction in NXR's grant funding since 2011. Additionally, different *Xenopu*s stock centers and individual researchers do not use a uniform cryopreservation method. Most of NXR's efforts are focused on creating new transgenic and mutant models.

Discussion

A participant asked about training programs at NXR. Dr. Horb explained that NXR hosts two 1-week workshops annually and a principal investigator meeting every other year. Another program allows laboratories to use NXR resources for a fee.

Dr. Horb clarified for a participant that the NXR maintains 70 cryopreserved samples; no other center maintains cryopreserved *Xenopus* species. He explained to another participant that NXR is funded with soft money, with 50% of its support derived from the Marine Biological Laboratory. It is difficult for NXR to hire and maintain knowledgeable technicians based on geography and cost of living. Dr. Varga noted that technician performance was crucial when cryopreservation was in its infancy, but as the protocols improve, technician performance is becoming less critical. Within the next few years, it will be much easier to teach replacement technicians the protocol.

Cryopreservation Status and Needs for the Axolotl
S. Randal Voss, University of Kentucky, Lexington, Kentucky

The *Ambystoma* Genetic Stock Center (AGSC) at the University of Kentucky maintains a historically significant collection of Mexican axolotls (*Ambystoma mexicanum*) and provides living materials from this resource in support of biomedical research nationally and internationally. Most notably, axolotls are studied because they are unique among vertebrates in being able to regenerate numerous tissues and body parts. The AGSC currently is not cryopreserving germplasm to preserve or re-derive lines and mutants but is interested in it. Cryopreservation methods and an overall cryopreservation strategy are needed because the AGSC does not have the infrastructure to serve as a repository for community-generated living stocks, including knockouts and transgenics.

Discussion

A participant asked whether the lines are strictly inbred. Dr. S. Randal Voss explained that the center maintains 11 different mutant lines with some genetic variation.

Dr. Voss clarified that AGSC does not have the resources to develop a specific cryopreservation protocol for axolotls but can perform cryopreservation onsite with an existing protocol. Cryopreservation would protect the existence of the research lines, which have been selected to breed and survive well in captivity. If the lines were to be lost, it would not be possible to duplicate them without cryopreserved samples because the wild-type axolotl is not the same.

Dr. Voss answered questions related to spawning, which is a time-consuming process. Each spawn produces one to 20 spermatophores and zero to 1,000 eggs. Fertilization must occur within 10 minutes of egg production. The process itself is highly efficient but time inefficient.

Augmenting the Preservation of Genetic Resources
Zoltan Varga, University of Oregon, Eugene, Oregon

The development of an improved sperm cryopreservation protocol has benefitted ZIRC operations and proven to be crucial for efficient import, management, and distribution of resources. Several areas, however, require continued attention and development of community-wide standards and methods. For example, potentially dangerous pathogens can be cryopreserved along with genetic resources. Hence, biosecurity protocols need to be developed that address the potential propagation of pathogens when cryopreserved resources are reactivated. Second, virtually no protocols exist specifically for the cryopreservation of zebrafish cell lines such as stem, somatic, or cancer. Current protocols rely on methods derived from cryopreservation protocols for bacteria or for cells from nonaquatic vertebrate species. Lastly, sperm cryopreservation and rederivation of lines necessitate the maintenance of live wild-type reference strains, which are needed to produce eggs for *in vitro* fertilization after sperm sample thawing. Thus, a portion of the genome is not preserved and continues to accumulate background mutations and undergoes loss of genetic diversity with every new generation. Zebrafish embryo or oocyte cryopreservation is urgently needed to reintroduce genetic diversity and reduce the frequency of background mutations in future generations of wild-type lines. In addition, restoring lines from cryopreserved embryos will maintain the molecular identity of recently sequenced reference strains.

Discussion

A participant asked about genetic drift. Dr. Varga explained that if the female portion of the genome (lineage) can be cryopreserved, then there will not be a discrepancy in the wild-type line accumulating sequence polymorphisms, variation, and drift away from the established sequence. The current method to avoid this is to thaw and back breed sperm. If large quantities of eggs or embryos could be preserved, it would be possible to directly go back to the current strain in the future. A participant noted that sperm contains essentially the entire genome, and it is possible to apply hormones and change the sex, further indicating the completeness of the genome. Some strategies to reconstitute lines include Dr. Blackburn's work to breed-back with the frozen sperm to generate the desired level of diversity. This can be done to shift the drift back. The participants discussed the different definitions of "drift" and agreed that resurrecting the genome from cryopreserved embryos is much faster and efficient than back breeding several generations with sperm.

A participant asked about somatic cell contamination. Dr. Varga explained that sperm stripped from the male should not have somatic cells present.

Cryopreservation in Viviparous Fishes (Xiphophorus spp.)
Ronald B. Walter, Texas State University, San Marcos, Texas

Xiphophorus backcross hybrids first were developed in 1927 as melanoma models, and additional *Xiphophorus* tumor models have been published since. The genus is used to explore cancer, sex determination, behavior genetics, hybridization, puberty, body size, and pigment cell biology. The

Xiphophorus Genetic Stock Center (XGSC) was established in 1930 and moved to Texas State University in 1992. The center is an NIH-supported national resource, with 24 of 26 *Xiphophorus* species available. The stock center maintains 53 pedigreed lines and has produced 29 distinct interspecies cross models. The various species developed in Mexico after being geographically separated, and interspecies hybridization produces fertile offspring. *Xiphophorus* sperm look different than the sperm of other species, and 2007 marked the first successful insemination with previously frozen sperm. Currently, approximately 20 lines have had enough sperm cryopreserved (i.e., at least 80 straws) to be considered appropriately backed up. These straws are banked at the USDA's National Animal Germplasm Program facility in Fort Collins, Colorado, under the direction of Dr. Harvey Blackburn. An additional 33 fish lines still need to be processed for sperm cryopreservation.

Cryopreservation of about one-third of the lines in the XGSC would allow the facility to save space and labor because these lines are being maintained for perpetuation of a single chromosome that could easily be regenerated from cryopreserved sperm. In addition, cryopreservation of germplasm from these species also is considered a conservation effort because the site locales for many species have been obliterated by urban expansion, and at least one line in the center is extinct in the wild (*X. couchianus*). The ability of these fish to produce fertile interspecies hybrids makes cryopreservation of sperm a viable mechanism to maintain the genetic content of species even if they become extinct in the wild.

Discussion

A participant asked about the use of a surrogate system. Dr. Ronald Walter explained that the XGSC had not attempted to apply a surrogate system to embryos because *Xiphophorus* are live-bearing with internal fertilization occurring within the female. Answering another question, he stated that the XGSC has carried several inbred lines (maintained via brother-sister mating) through more than 100 generations with no apparent problems for fish health, at least within the culture conditions used at XGSC.

Cryopreservation Challenges for the National Resource for Aplysia
Michael Schmale, University of Miami, Miami, Florida

Aplysia californica, the California sea hare, is an ideal system for studies of neural circuits, the cellular basis of memory and learning, and changes associated with aging in an annual animal. The National Resource for *Aplysia* breeds, rears, and ships approximately 20,000 *Aplysia* per year at all life stages to U.S. and worldwide laboratories. It is the only facility of its kind and the only source of animals in early life stages. Cryopreservation would be advantageous for this species to create an archive of specific crosses for use over multiple years, save inbred lines for future genome studies, and assist future efforts to create transgenic lines. Several challenges, however, are associated with cryopreservation of *Aplysia*. Because the animal uses internal fertilization, frozen eggs or sperm cannot be used for *in vitro* fertilization. The embryos are encased in a double-layered egg capsule in addition to the chorion before being released, and it is very difficult to penetrate this capsule. Because the embryos do not survive removal from the egg case, no simple method exists to freeze isolated embryos. Fortunately, though, hermaphroditic (non-self-fertilizing) animals breed constantly, making it easy to obtain millions of eggs, which hatch in approximately 7 days. Also, the egg cases are tough and easy to handle, but it will be necessary to identify a method to manipulate the eggs within the capsule or viably remove them from the capsule. The question is whether it is possible to cryopreserve newly hatched veliger larvae or trochophore larvae, the latter of which are formed 72 hours post-fertilization and "swim" inside the egg case. Another question is which life stages could be cryopreserved and survive, particularly considering *Aplysia*'s complex biology. Cryopreserving the larvae would be advantageous to avoid having to surgically implant cryopreserved sperm back into the animal.

Discussion

A participant asked about other existing models. Dr. Michael Schmale explained that *Aplysia* are gastropods, and other gastropod models for neurobiology exist, although *Aplysia* is by far the most widely used. He described two approaches to transgenics. The first would be to inject DNA into the egg capsule right after it has been laid (prior to first cleavage) and then determine whether any injected material will enter the eggs. The second would be to inject DNA into the testis, which would require surgery. Either approach could be combined with electroporation across the tissue.

Session 4: Summary and Recommendations

Session Chairs: Mary Hagedorn, Smithsonian and Conservation Biology Institute and Hawaii Institute of Marine Biology, Kaneohe, Hawaii, and Ronald B. Walter, Texas State University, San Marcos, Texas

The participants discussed the possible areas in which to provide specific, actionable recommendations to ORIP, identifying three clusters dealing with scope, scale, and approach. The participants agreed that addressing only one of these would not be enough; all three must be addressed to be successful. The three clusters are as follows:

1. Technology development in cryopreservation samples
   a. Embryo cryopreservation
   b. Other sample (e.g., stem cells, sperm) cryopreservation

2. Improvement in cryopreservation mechanics (i.e., engineering technology and solutions)
   a. Improvements in microfluidic handling of embryos in cryopreservation protocols
   b. Improvements in cryopreservation efficiency and pipelines (e.g., automation, smaller sample volumes)

3. Establishment of a centralized center of cryopreservation expertise
   a. Fellowship/training in cryopreservation
   b. Dedicated expertise in developing aquatic models, protocols, and pipelines
   c. Development of customized protocols and pipelines
   d. Standardized digital record keeping, working back-up samples, long-term storage, and so forth

Prior to the meeting, participants were asked to respond to a survey. Dr. Varga gave an overview of the survey questions and results, which are provided as Appendix C. He commented that the survey should be reduced to 10 questions when it is developed for a wider audience, and it should ask for suggestions for short- and long-term actions.

The goal of the discussion was to understand exactly what has been done and how to address existing challenges; some systems may be more easily addressed than others, and each aquatic model has specific needs. Although many of the previous workshop discussions focused on cryopreservation more than on viability after thawing, the question is whether animals grow and live a normal life following thawing. The participants agreed that revival must be considered, and post-fertilization animals must be evaluated. Cryopreservation of somatic cells also must be considered; some zoos are cryopreserving somatic cells, and even cryopreserved blood cells may be useful. The ability to cryopreserve oocytes is a technical issue related to developing the right protocol. Eggs also should be preserved even if *in vitro* fertilization is not successful.

The participants discussed harmonization and standardization across and within communities, respectively, with the priority being to identify resources to start this process. This will be especially helpful for laboratories that are not able to justify the cost of investing in cryopreservation equipment. "Tactical implementation" that allows sharing across communities and access to all laboratories is important. This could include establishing a center that allows laboratory personnel to visit and use the resources or supporting center personnel to visit laboratories and provide training and expertise. The four tactical strategies for laboratories are to (1) acquire and use the technology in-house, (2) invite personnel from a center to the laboratory to perform cryopreservation or training, (3) send material to a center for cryopreservation, or (4) send personnel to a center to receive training on protocols that do not require the laboratory to purchase special equipment or technologies. Community-based resources can be established that are centralized or distributed, and a culture of collaboration must be fostered.

A centralized unit where all aquatic model system communities can go to acquire expertise is desired. This unit must consistently apply standards to all aquatic resources and harmonize protocols. Storage would occur at individual resources centers, many of which must distribute their aquatic resources. Programmatic development is critical. The unit also may need to possess husbandry capabilities so that those professionals being trained at the center can work on their specific models rather than learning in a theoretical manner. A board of directors with expertise in this area should advise the centralized unit.

Knowledge loss is a concern, so it would be useful to develop a video to ensure that the knowledge and expertise are available over time despite retirement and mortality. Training must be specific to each species and model. The community also must consider and plan for future technical, staff, and knowledge needs. The ability to network at workshops and meetings also is critical.

The group discussed establishing this workshop as an annual workshop. Dr. Michael Chang (ORIP, NIH) commented that a follow-up workshop potentially could be planned in 2 years to examine the status of the effort and recalibrate efforts as necessary. Participants noted other meeting opportunities. A cryopreservation session could be added to the Aquatic Animal Models of Human Disease Conference, and workshop participants could attend the Annual Meeting of the Society for Cryobiology, which is held in the summer. The participants agreed that it would be helpful to develop a calendar of important, advantageous meetings.

Ultimately, the participants developed the following recommendations:

- Establish a comprehensive, centralized unit ("hub") to programmatically develop training for and documentation of cryopreservation of aquatic model systems. This will include the development of species-specific protocols and approaches, outreach programs, community development and standardization, and the training of the next generation of aquatic cryopreservation experts.

- Develop a call for innovative technical advancements that will increase the reliability, reproducibility, simplicity, throughput, and efficiency of the cryopreservation process, including vitrification and pipelines for embryos, eggs, sperm, oocytes, stem cells, and somatic cells of all aquatic species. This recommendation encompasses basic cryopreservation knowledge and engineering technology, such as microfluidics and processing technologies.

- Implement mechanisms that allow stock centers to increase their planning, personnel, and ability to secure genetic resources and develop their own repositories as well as allow them to interact within an integrated, comprehensive repository network for aquatic model species.

Appendix A: Workshop Agenda

Cryopreservation of Aquatic Biomedical Models Workshop
Agenda

Objectives:

- Assess the status of germplasm cryopreservation in various aquatic models.
- Identify the obstacles, opportunities, and priorities that may address the need for improved methods.
- Evaluate novel and emerging research and technologies that might lead to the successful preservation of other "germplasm format" (sperm, embryo, oocyte, ovarian tissue, testicular tissue, embryonic stem cells).

Organizing Committee:

Mary Hagedorn (Chair), Smithsonian Conservation Biology Institute (SCBI) and Hawaii Institute of Marine Biology (HIMB), Kaneohe, Hawaii
John Bischof, University of Minnesota, Minneapolis, Minnesota
Harvey Blackburn, U.S. Department of Agriculture, National Animal Germplasm Program, Fort Collins, Colorado
Michael Chang, Office of Research Infrastructure Programs (ORIP), Division of Program Coordination, Planning, and Strategic Initiatives (DPCPSI), National Institutes of Health (NIH), Bethesda, MD
Miguel Contreras, ORIP, DPCPSI, NIH, Bethesda, Maryland
Stuart Meyers, University of California, Davis, Davis, California
Terrence R. Tiersch, Aquatic Germplasm and Genetic Resources Center, Louisiana State University Agricultural Center (LSUAC), Baton Rouge, Louisiana
Zoltan Varga, Zebrafish International Research Center, University of Oregon, Eugene, Oregon
Desiree Vonkollmar, ORIP, DPCPSI, NIH, Bethesda, Maryland
Ronald B. Walter, *Xiphophorus* Genetic Stock Center, Texas State University, San Marcos, Texas
Sige Zou, ORIP, DPCPSI, NIH, Bethesda, Maryland

Revised Workshop Schedule (Winter Storm Helena affected the area, causing severe weather):

January 7, 2017

3:15 – 3:30 p.m. **Registration**

3:30 – 3:45 p.m. **Introduction and Welcome**

Mary Hagedorn, SCBI/HIMB, Kaneohe, HI
Miguel Contreras, ORIP, NIH, Bethesda, MD

3:45 – 5:15 p.m. **Session 1**: **Opportunities for Cryopreservation of Aquatic Species: Eggs, Embryos, Reproductive Tissues, and Methods**

Session Chair: Michael Chang, ORIP, DPCPSI, NIH, Bethesda, MD

Development of a Universal Platform for Aquatic Germplasm Cryopreservation: John Bischof, University of Minnesota, Minneapolis, MN; and Mary Hagedorn, SCBI/HIMB, Kaneohe, HI

Session 1 (continued)

Lyopreservation of Sperm: Sankha Bhowmick, University of Massachusetts Dartmouth, Dartmouth, MA.

Practical Method for Cryopreservation of Medaka Sperm and Its Application to Other Aquatic Models: Kiyoshi Naruse, National Institute for Basic Biology, Aichi, Japan

Production of Viable Trout Offspring Derived From Frozen Testis Via Germ Cell Transplantation: Goro Yoshizaki, Tokyo University of Marine Science and Technology, Tokyo, Japan

Practical Applications of Somatic Cell Nuclear Transfer in Zebrafish: Jose Cibelli, Michigan State University, East Lansing, MI

Discussion: Speakers and attendees

5:15 – 6:30 p.m. **Session 2: Reproducibility and Standardization for Repository Development and Throughput/Scalability Technologies**

Session Chair: John Bischof, University of Minnesota, Minneapolis, MN

Reproducibility, Quality Control, and Standardization: Terrence Tiersch, LSUAC, Baton Rouge, LA

Microfabricated Devices for Standardization, Reproducibility, and Throughput of Sperm Cryopreservation: Todd Monroe, LSU, Baton Rouge, LA

Can Microfluidic Platforms for Droplets and Bubbles Enable High Throughput Cryopreservation? Cari Dutcher, University of Minnesota, Minneapolis, MN

Genetic Resource Preservation and Management at the Zebrafish International Resource Center: Zoltan Varga, University of Oregon, Eugene, OR

Discussion: Speakers and attendees

6:30 – 8:00 p.m. **Opening Reception for Aquatic Animal Models of Human Disease Conference**

8:00 – 10:00 p.m. **Session 3: Perspectives From Resource Centers on Current Cryopreservation Status and Roadblocks**

Session Chair: Terrence Tiersch, LSUAC, Baton Rouge, LA

Implementing Cryopreservation in Xenopus for the National Xenopus Resource: Marko Horb, Marine Biological Laboratory, Woods Hole, MA

Cryopreservation Status and Needs for the Axolotl: S. Randal Voss, University of Kentucky, Lexington, KY

Session 3 (continued)

Augmenting the Preservation of Genetic Resources: Zoltan Varga, University of Oregon, Eugene, OR

Cryopreservation in Viviparous Fishes (Xiphophorus spp.): Ronald B. Walter, Texas State University, San Marcos, TX

Cryopreservation Challenges for the National Resource for Aplysia: Michael Schmale, University of Miami, Miami, FL

Panel Discussion Focus:

Current state of cryopreservation efforts for various aquatic species.

Identification of current road blocks in cryopreservation and the implications for translational research.

Technological needs for overcoming identified road blocks for cryopreservation.

Wrap up, summary, and recommendations.

January 8, 2017

8:00 – 9:30 a.m. **Session 4: Summary and Recommendations**

Session Chair: Mary Hagedorn, SCBI/HIMB, Kaneohe, HI; and Ronald B. Walter, Texas State University, San Marcos, TX

Questions to Focus Discussion:

What are the needs/gaps for cryopreserving aquatic species? What is required to fulfill these needs/gaps (e.g., basic research, resources, technologies, training)?

What recommendations can be made to ORIP regarding reproductive and cryopreservation research relative to aquatic models? Specific focus for recommendations include:

- Specific Topical Research
- Strategies for Funding
- Community-Based Resources Needs for Aquatic Resources (all models)

9:30 – 10:30 a.m. **Session Summary**

Organizing Committee Members

Appendix B: Workshop Participants

Cryopreservation of Aquatic Biomedical Models Workshop
List of Participants

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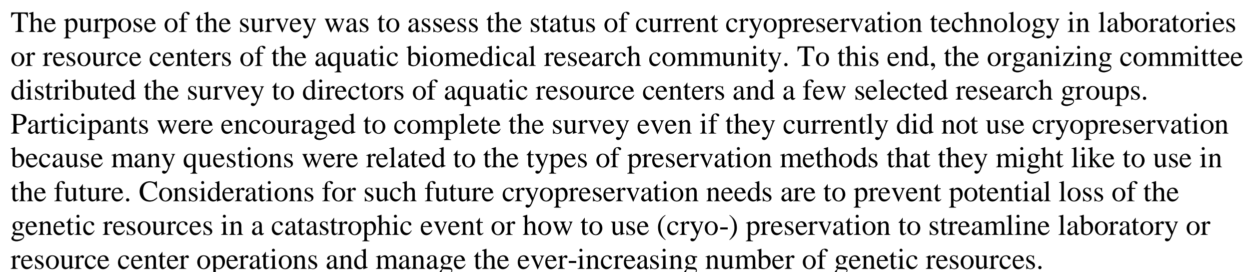
Appendix C: Protection of Essential Genetic Resources: Survey for Resource Centers and Research Community



The purpose of the survey was to assess the status of current cryopreservation technology in laboratories or resource centers of the aquatic biomedical research community. To this end, the organizing committee distributed the survey to directors of aquatic resource centers and a few selected research groups. Participants were encouraged to complete the survey even if they currently did not use cryopreservation because many questions were related to the types of preservation methods that they might like to use in the future. Considerations for such future cryopreservation needs are to prevent potential loss of the genetic resources in a catastrophic event or how to use (cryo-) preservation to streamline laboratory or resource center operations and manage the ever-increasing number of genetic resources.

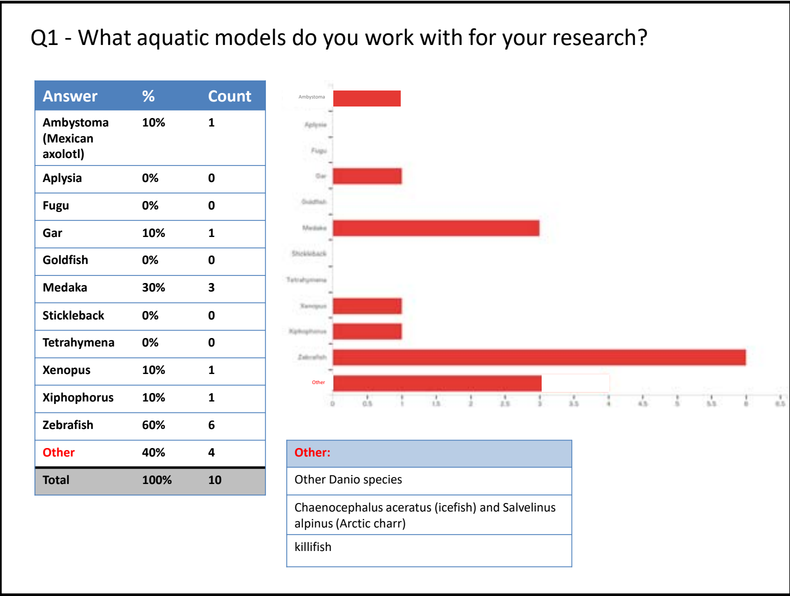
The survey collected information on the aquatic species that currently are used and what type of genetic resources and storage and exchange solutions exist (Q1–Q10). Additional questions explored the shortcomings and risks of present repositories and explored what preservation technologies, storage methods, and additional genetic resources are needed to enhance the use of and advance aquatic models for biomedical research. Importantly, all aquatic resource centers indicated that cryopreservation technology is urgently needed to back up valuable genetic resources for their communities to (1) mitigate catastrophic loss (Q7) and (2) advance the research value of their aquatic organisms (Q21). Question 13 indicated that sperm cryopreservation is used with moderate to excellent success for 50% of the respondents; however, most aquatic species completely lack cryopreservation of oocytes and ovarian, testicular, embryonic, and adult somatic (stem) cells, all of which are preserved successfully and extensively used in mammalian biomedical research and enhance the palette of genetic and reproductive experimental capabilities in these species. At the minimum, all resource centers wanted to utilize sperm cryopreservation (Q14), and a majority expressed the need to also cryopreserve embryos (90%) or oocytes (70%). Respondents unanimously agreed to implement and utilize practical cryopreservation technology at the various aquatic repositories in the future (Q15). With cryopreservation, several advanced supporting technologies also need to be developed, including cell manipulations, microinjection, microfluidics, and information systems. The survey and subsequent discussion with resource center directors indicated that support for these supporting technologies also is urgently needed (Q17–Q19).

The results of this survey, based on 10 received responses, were presented and discussed during the workshop. Hopefully, the results from this survey will help the workshop participants to better understand the current practices and future needs of current or additional preservation methods in the aquatic research community. In the long term, this information will ensure that valuable genetic resources will be protected and available in various formats to future generations of researchers.

Survey Report

Protection of Essential Genetic Resources: Survey for Resource Centers and Research Community

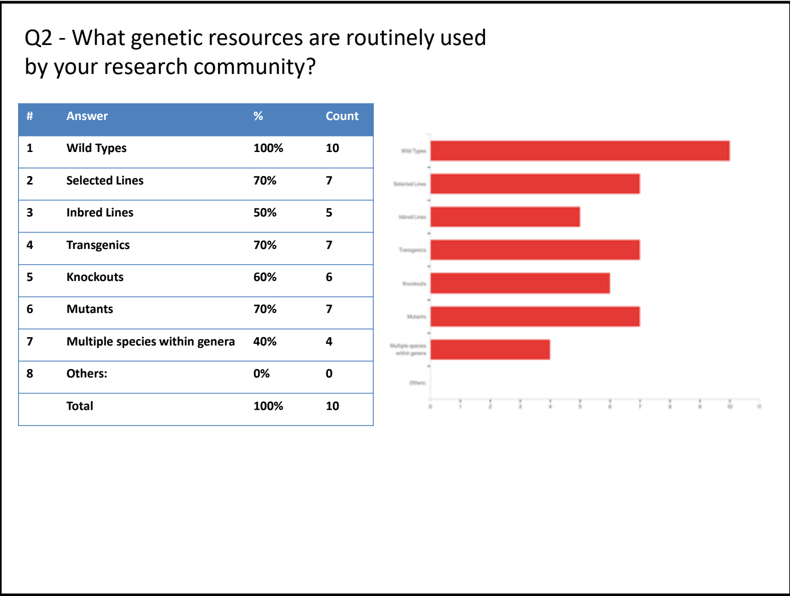
January 2nd 2017, 11:51 am PST



Q1 - What aquatic models do you work with for your research?

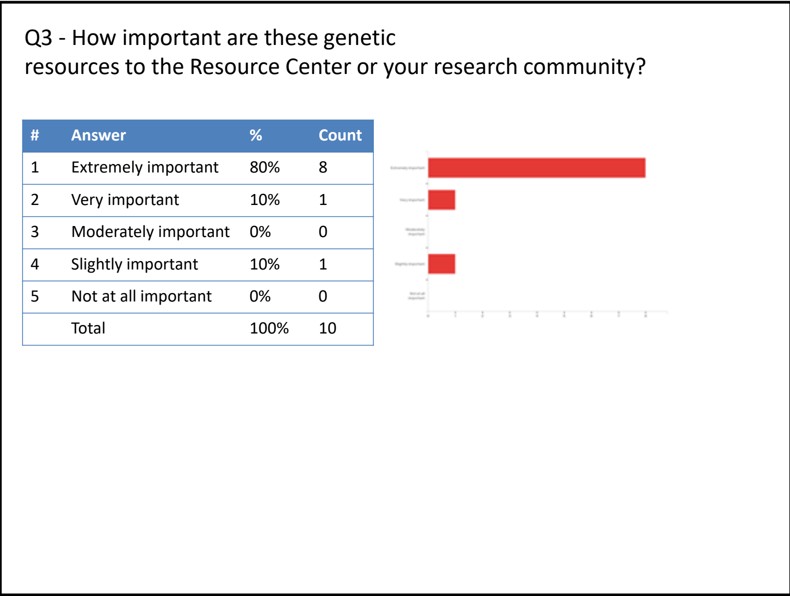
Answer	%	Count
Ambystoma (Mexican axolotl)	10%	1
Aplysia	0%	0
Fugu	0%	0
Gar	10%	1
Goldfish	0%	0
Medaka	30%	3
Stickleback	0%	0
Tetrahymena	0%	0
Xenopus	10%	1
Xiphophorus	10%	1
Zebrafish	60%	6
Other	40%	4
Total	100%	10

Other:
Other Danio species
Chaenocephalus aceratus (icefish) and Salvelinus alpinus (Arctic charr)
killifish



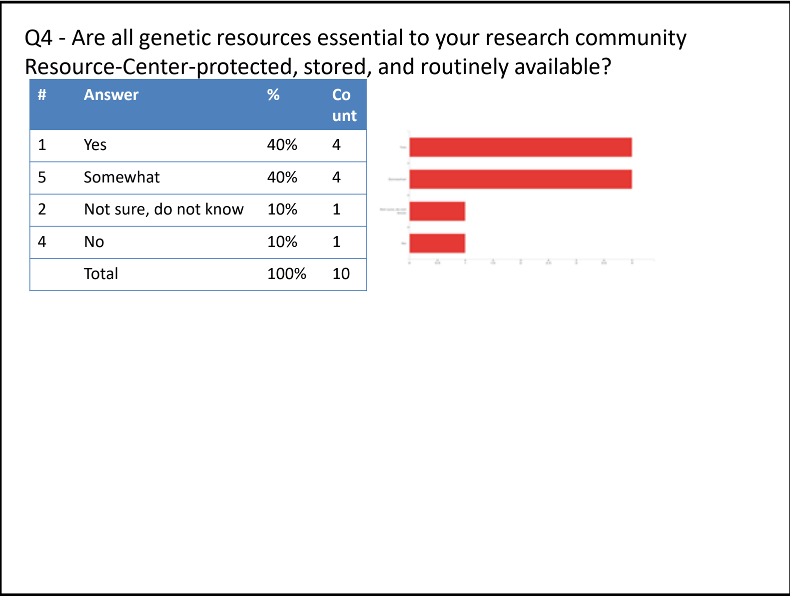
Q2 - What genetic resources are routinely used by your research community?

#	Answer	%	Count
1	Wild Types	100%	10
2	Selected Lines	70%	7
3	Inbred Lines	50%	5
4	Transgenics	70%	7
5	Knockouts	60%	6
6	Mutants	70%	7
7	Multiple species within genera	40%	4
8	Others:	0%	0
	Total	100%	10



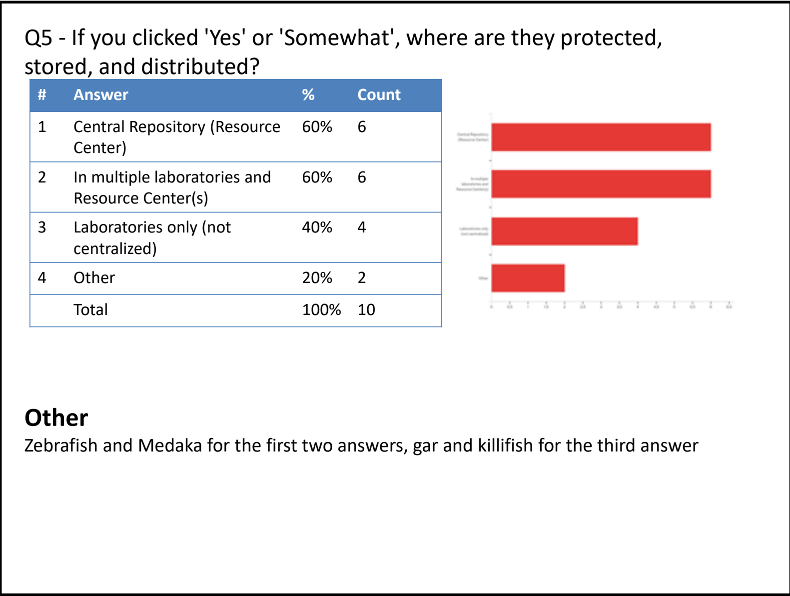
Q3 - How important are these genetic resources to the Resource Center or your research community?

#	Answer	%	Count
1	Extremely important	80%	8
2	Very important	10%	1
3	Moderately important	0%	0
4	Slightly important	10%	1
5	Not at all important	0%	0
	Total	100%	10



Q4 - Are all genetic resources essential to your research community Resource-Center-protected, stored, and routinely available?

#	Answer	%	Count
1	Yes	40%	4
5	Somewhat	40%	4
2	Not sure, do not know	10%	1
4	No	10%	1
	Total	100%	10

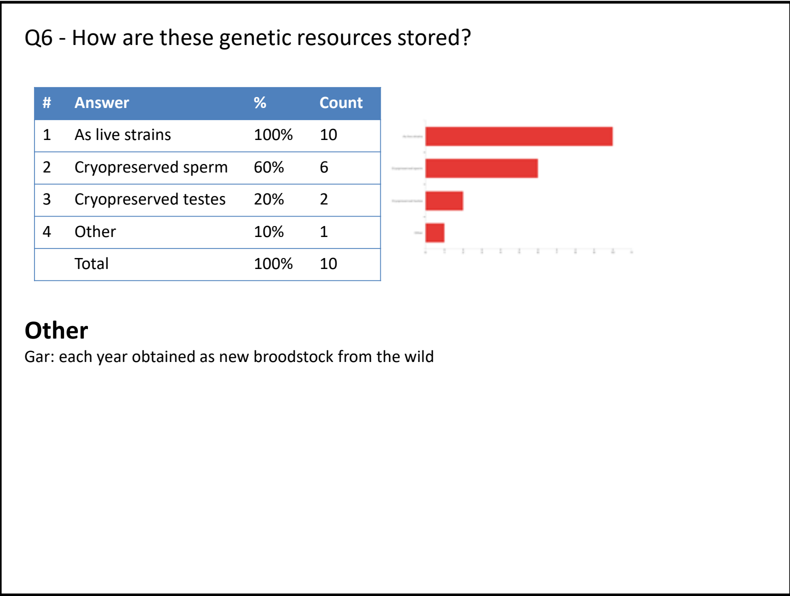


Q5 - If you clicked 'Yes' or 'Somewhat', where are they protected, stored, and distributed?

#	Answer	%	Count
1	Central Repository (Resource Center)	60%	6
2	In multiple laboratories and Resource Center(s)	60%	6
3	Laboratories only (not centralized)	40%	4
4	Other	20%	2
	Total	100%	10

Other

Zebrafish and Medaka for the first two answers, gar and killifish for the third answer

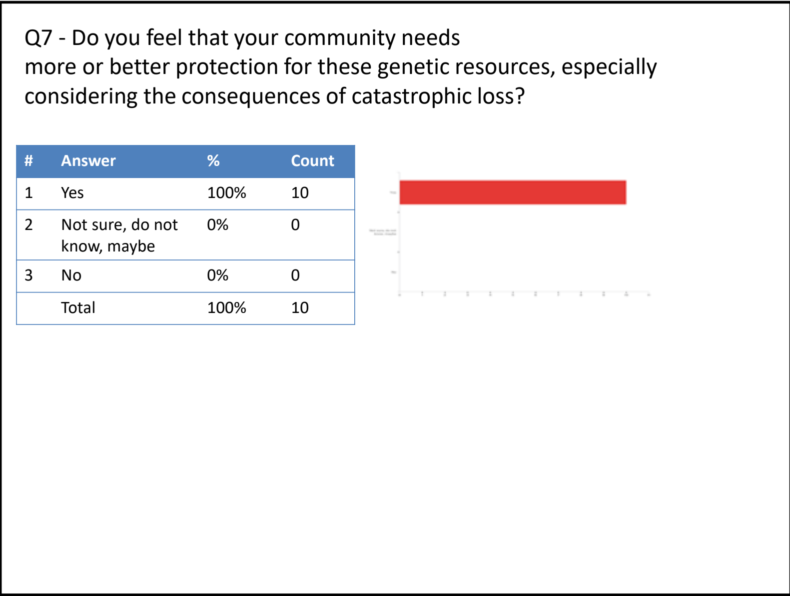


Q6 - How are these genetic resources stored?

#	Answer	%	Count
1	As live strains	100%	10
2	Cryopreserved sperm	60%	6
3	Cryopreserved testes	20%	2
4	Other	10%	1
	Total	100%	10

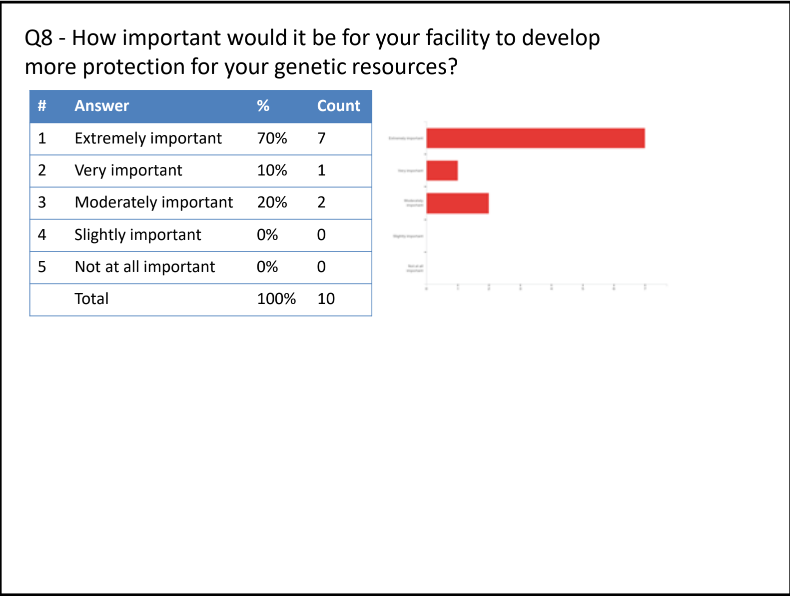
Other

Gar: each year obtained as new broodstock from the wild



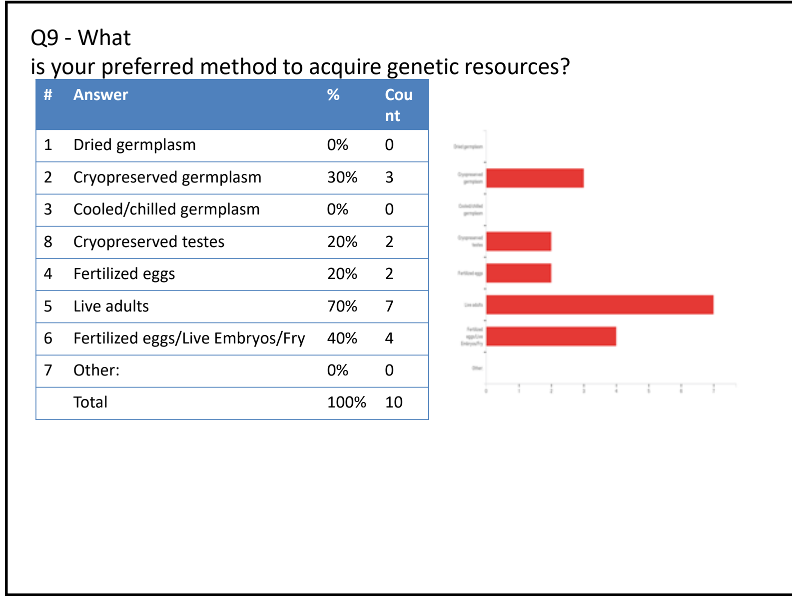
Q7 - Do you feel that your community needs more or better protection for these genetic resources, especially considering the consequences of catastrophic loss?

#	Answer	%	Count
1	Yes	100%	10
2	Not sure, do not know, maybe	0%	0
3	No	0%	0
	Total	100%	10



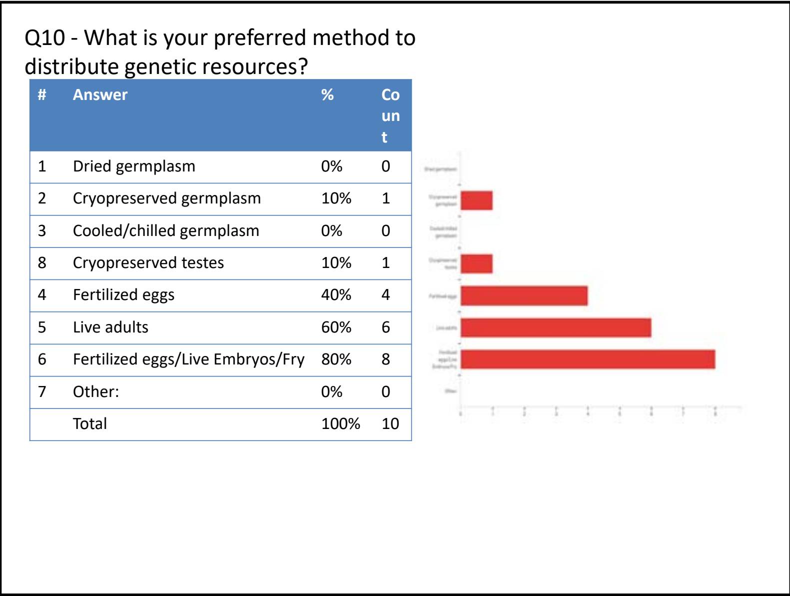
Q8 - How important would it be for your facility to develop more protection for your genetic resources?

#	Answer	%	Count
1	Extremely important	70%	7
2	Very important	10%	1
3	Moderately important	20%	2
4	Slightly important	0%	0
5	Not at all important	0%	0
	Total	100%	10



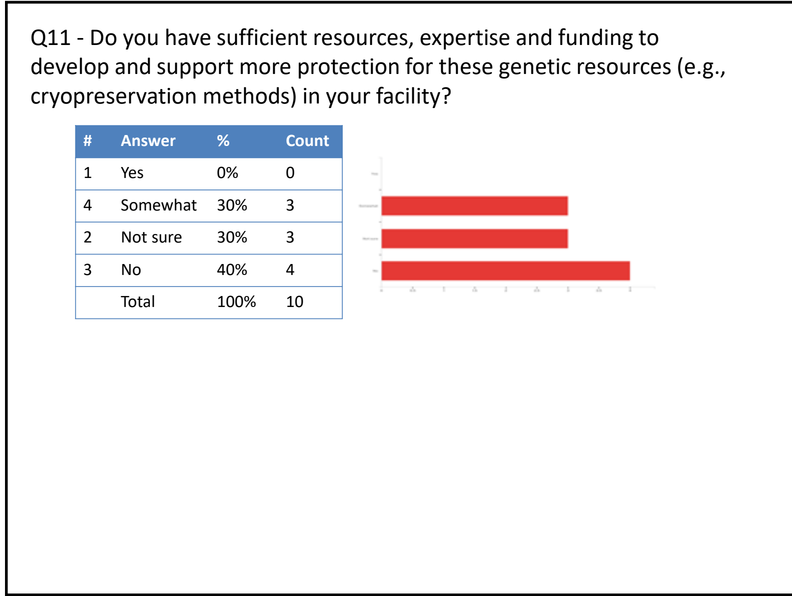
Q9 - What is your preferred method to acquire genetic resources?

#	Answer	%	Count
1	Dried germplasm	0%	0
2	Cryopreserved germplasm	30%	3
3	Cooled/chilled germplasm	0%	0
8	Cryopreserved testes	20%	2
4	Fertilized eggs	20%	2
5	Live adults	70%	7
6	Fertilized eggs/Live Embryos/Fry	40%	4
7	Other:	0%	0
	Total	100%	10



Q10 - What is your preferred method to distribute genetic resources?

#	Answer	%	Count
1	Dried germplasm	0%	0
2	Cryopreserved germplasm	10%	1
3	Cooled/chilled germplasm	0%	0
8	Cryopreserved testes	10%	1
4	Fertilized eggs	40%	4
5	Live adults	60%	6
6	Fertilized eggs/Live Embryos/Fry	80%	8
7	Other:	0%	0
	Total	100%	10



Q11 - Do you have sufficient resources, expertise and funding to develop and support more protection for these genetic resources (e.g., cryopreservation methods) in your facility?

#	Answer	%	Count
1	Yes	0%	0
4	Somewhat	30%	3
2	Not sure	30%	3
3	No	40%	4
	Total	100%	10

Q12 - What type of germplasm does your laboratory or center currently cryopreserve?

#	Answer	%	Count
2	Oocytes	0%	0
1	Sperm	50%	5
4	Embryos	10%	1
3	Ovarian stem cells	0%	0
8	Testicular stem cells	10%	1
5	Embryonic stem cells	0%	0
7	Gonadal tissue slices	0%	0
6	Adult stem cells	0%	0
10	Testes	10%	1
11	Ovaries	0%	0
12	Other (live lines)	40%	4

Other [in keywords: please specify the type of germplasm, and whether the s...
only live lines
None
none
we do not cryopreserve germ plasm

Q13 - What status is this cryopreservation technology as practiced in your laboratory or resource center?

[select one per row; if other technologies are available, please list in Q23 and indicate status]

#	Question	Excellent, ≥80% Post-thaw Viability		Quite reliable, 79%-50% Post-thaw Viability		Adequate, 49%-20% Post-thaw Viability		Not very reliable, ≤20% Post-thaw Viability		Not available		Total
2	Oocytes	0%	0	0%	0	0%	0	0%	0	**100%**	10	10
1	**Sperm**	**10%**	1	**20%**	2	**30%**	3	0%	0	40%	4	10
4	**Embryos**	0%	0	0%	0	0%	0	**10%**	1	90%	9	10
3	Ovarian stem cells	0%	0	0%	0	0%	0	0%	0	**100%**	10	10
8	**Testicular stem cells**	0%	0	**10%**	1	0%	0	0%	0	90%	9	10
5	Embryonic stem cells	0%	0	0%	0	0%	0	0%	0	**100%**	10	10
7	Gonadal tissue slices	0%	0	0%	0	0%	0	0%	0	**100%**	10	10
6	Adult stem cells	0%	0	0%	0	0%	0	0%	0	**100%**	10	10
12	**Testes**	0%	0	**10%**	1	0%	0	0%	0	90%	9	10
13	Ovaries	0%	0	0%	0	0%	0	0%	0	**100%**	10	10

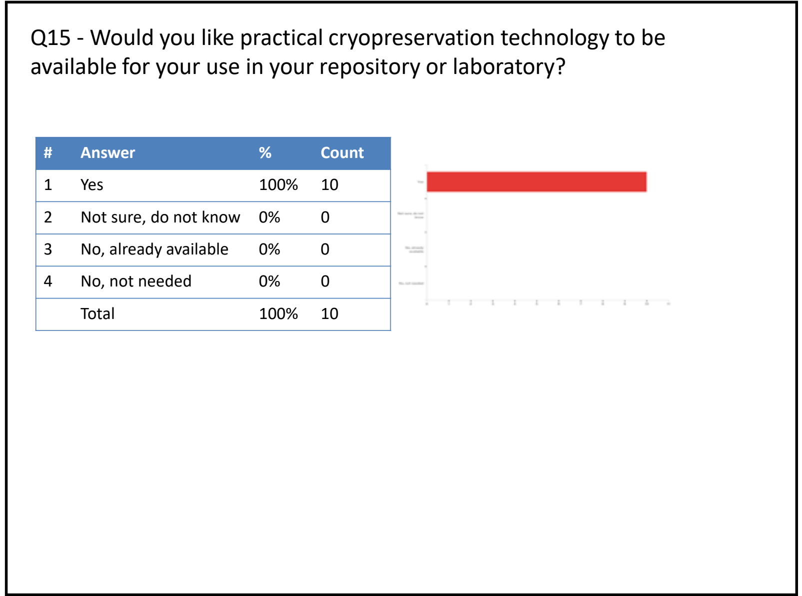
Q13 - What status is this cryopreservation technology as practiced in your laboratory or resource center?

[select one per row; if other technologies are available, please list in Q23 and indicate status]

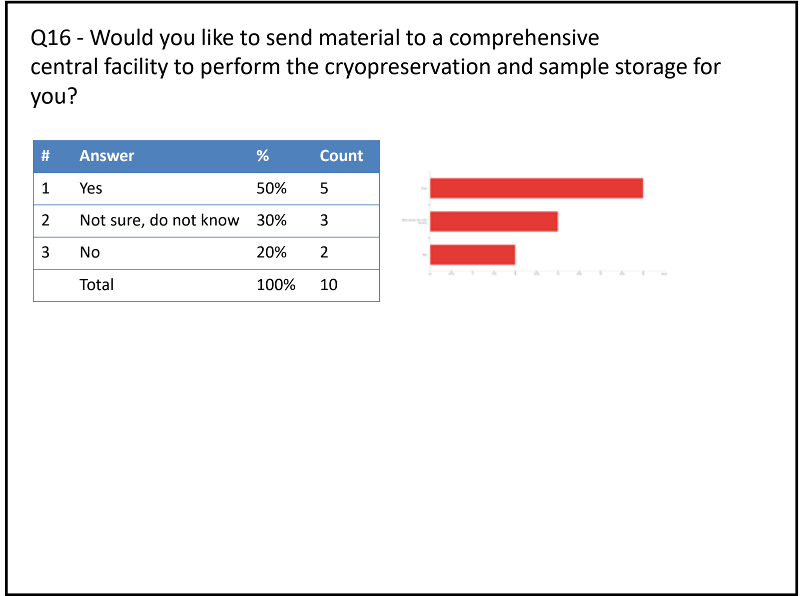
Excellent, ≥80% Post-thaw Viability
Quite reliable, 79%-50% Post-thaw Viability
Adequate, 49%-20% Post-thaw Viability
Not very reliable, ≤20% Post-thaw Viability
Not available
0 1 2 3 4 5 6 7 8 9 10 11
Oocytes
Sperm
Embryos
Ovarian stem cells
Testicular stem cells
Embryonic stem cells
Gonadal tissue slices
Adult stem cells
Testes
Ovaries

Q14 - What type of cryopreservation would you hope to have routinely available for your community (your research) in the future?

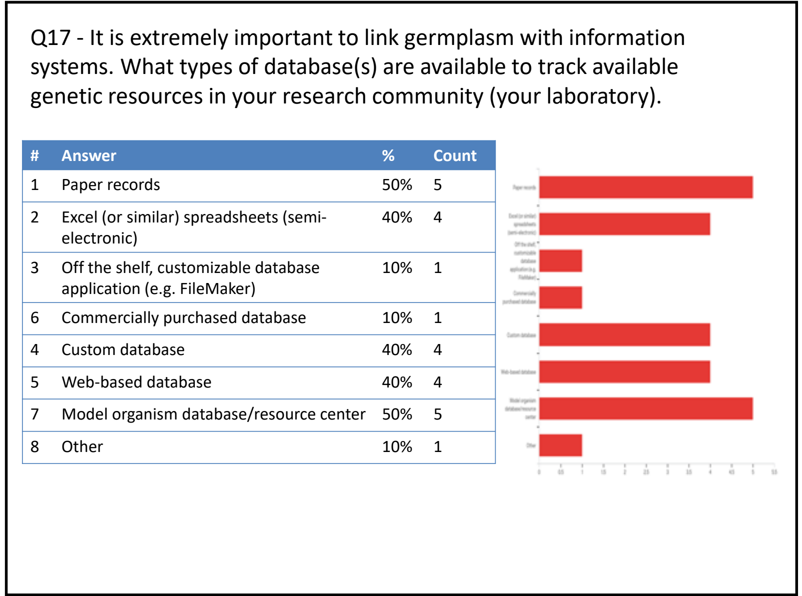
#	Answer	%	Count
2	Oocytes	70%	7
1	Sperm	100%	10
10	Embryos	90%	9
3	Ovarian stem cells	20%	2
4	Testicular stem cells	20%	2
5	Embryonic stem cells	40%	4
7	Gonadal tissue slices	10%	1
6	Adult stem cells	50%	5
9	Other (please specify)	0%	0


Q15 - Would you like practical cryopreservation technology to be available for your use in your repository or laboratory?

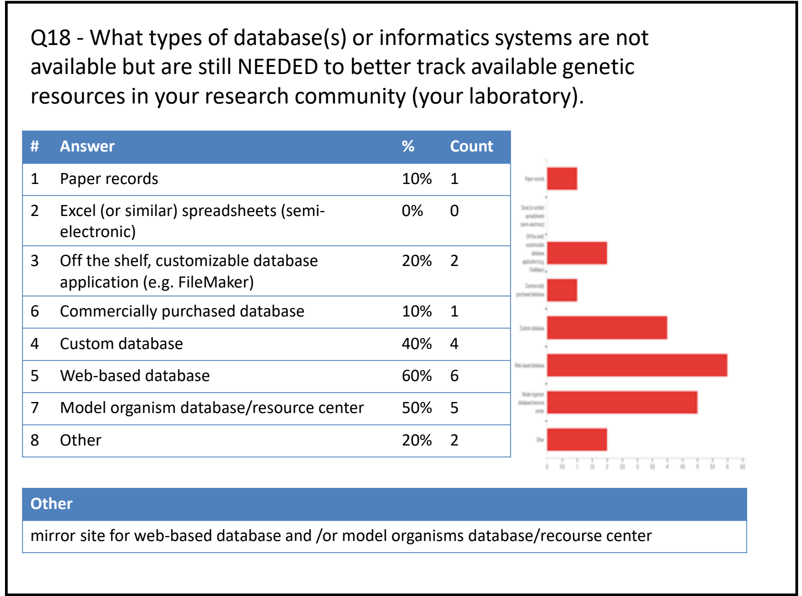
#	Answer	%	Count
1	Yes	100%	10
2	Not sure, do not know	0%	0
3	No, already available	0%	0
4	No, not needed	0%	0
	Total	100%	10


Q16 - Would you like to send material to a comprehensive central facility to perform the cryopreservation and sample storage for you?

#	Answer	%	Count
1	Yes	50%	5
2	Not sure, do not know	30%	3
3	No	20%	2
	Total	100%	10


Q17 - It is extremely important to link germplasm with information systems. What types of database(s) are available to track available genetic resources in your research community (your laboratory).

#	Answer	%	Count
1	Paper records	50%	5
2	Excel (or similar) spreadsheets (semi-electronic)	40%	4
3	Off the shelf, customizable database application (e.g. FileMaker)	10%	1
6	Commercially purchased database	10%	1
4	Custom database	40%	4
5	Web-based database	40%	4
7	Model organism database/resource center	50%	5
8	Other	10%	1


Q18 - What types of database(s) or informatics systems are not available but are still NEEDED to better track available genetic resources in your research community (your laboratory).

#	Answer	%	Count
1	Paper records	10%	1
2	Excel (or similar) spreadsheets (semi-electronic)	0%	0
3	Off the shelf, customizable database application (e.g. FileMaker)	20%	2
6	Commercially purchased database	10%	1
4	Custom database	40%	4
5	Web-based database	60%	6
7	Model organism database/resource center	50%	5
8	Other	20%	2

Other
mirror site for web-based database and /or model organisms database/recourse center

Q19 - Several advanced supporting technologies (such as microinjection, intracytoplasmic sperm injection, vitrification, laser warming/thawing, or 3-D printing) could enhance cryopreservation of genetic resources. Which of the following criteria for these supporting technologies will add value for your research?
[rank by by drag and drop criteria up or down; most important = 1/top, least important =6/bottom]

#	Question	1		2		3		4		5		6		Total
1	Reproducibility	50%	4	0%	0	37.50%	3	0%	0	12.50%	1	0%	0	8
2	Simplicity	12.50%	1	25%	2	12.50%	1	50%	4	0%	0	0%	0	8
3	Cost-efficiency	0%	0	12.50%	1	0%	0	37.50%	3	50%	4	0%	0	8
4	Time-efficiency	12.50%	1	0%	0	50%	4	12.50%	1	25%	2	0%	0	8
5	Reliability	25%	2	62.50%	5	0%	0	0%	0	12.50%	1	0%	0	8
6	Other criteria	0%	0	0%	0	0%	0	0%	0	0%	0	100%	8	8

Reproducibility
Simplicity
Cost-efficiency
Time-efficiency
Reliability
Other criteria

Reliability > Reproducibility

Reproducibility = Simplicity = Cost-efficiency = Time efficency

Q20 - Would your research benefit from increased reproducibility if reliable cryopreserved genetic resources were routinely available?

#	Answer	%	Count
1	Would not benefit / remain the same	0%	0
2	Would benefit moderately	10%	1
3	Would benefit	30%	3
4	Would benefit significantly	60%	6
	Total	100%	10

Q21 - Would developing better cryopreservation methodologies advance the role of the aquatic model organism(s) which you selected in Question 1 for translational research?

#	Answer	%	Count
1	Would not advance / remain the same	0%	0
3	Would advance moderately	20%	2
4	Would advance	40%	4
5	Would advance significantly	40%	4
	Total	100%	10

Q22 - Which areas of biomedical research would be advanced or supported by combining cryopreservation of genetic resources with your research or within your research community?

Which areas of translational medicine would be advanced by combining cryopr...
The ability to cryopreserve embryos, oocytes and other germ plasm beyond just sperm would help preserve reference strains thereby guarding against background mutation within both wild type and mutant strains.
Comparative genetics, tumor etiology, genetic regulation
The axolotl is a model in the areas of regenerative biology and stem cell research. Additionally, there are mutants that are of interest to researchers in other areas, some which are difficult to propagate because of lethality/sterility.
All type of biomedical researches using disease models are advanced by cryopreservationist of genetic resources. Because Good disease models are depend on their physiological properties according to their genetic background.
Translational Medicine; functional testing of homologues of human diseases/conditions

Q23 - Do you have any other suggestions or concerns regarding these topic areas or protection of genetic resources?
[optional, keywords/examples, up to 750 characters]

Do you have any other comments, suggestions, concerns, or thoughts regardin...
Creating high-throughput reliable cryopreservation protocols for zebrafish germplasm beyond sperm could help revolutionize the storage and maintenance of genetic resources for zebrafish research.
Better integration of genetic resources from many different models.
There is need to protect existing and future transgenic and knockout axolotl lines by cryopreservation. Currently, there is not a plan within our community to bank newly recreated lines and mutants so that they can be made widely available. Given the cost and time to maintain axolotls, which are relatively large, long-lived, and slow-developing, it is concerning that valuable new lines and mutants will not be shared, or will be lost or not maintained after they are created by investigators.
Practical workshop of cryopreservation for various aquatic organisms and the international meeting of cryopreservation and its theoretical basis (theory of verification, physical propriety of water etc)
Funding of current and additional cryopreservation methodologies is critical, because it will improve preservation of genetic resources as published and minimize genetic drift and background mutations, which reduce future reproducibility of genetic research.