

2020 Axolotl Community White Paper

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Section I: Summary

- A. The Axolotl is an essential animal model for biomedical research, especially in the areas of regenerative biology and medicine. The laboratory Axolotl traces its ancestry back to a collection of 34 Axolotls that were shipped to Paris in 1863 from aquatic habitats near present day Mexico City. Over the next few decades, Axolotls were propagated in laboratories across Europe and used to study questions in development and evolution, and these studies helped originate the field of experiment zoology. During the 20th century, Axolotls factored prominently in studies of embryonic and post-embryonic development, sex determination, cloning, and tissue regeneration. Today, Axolotls are attracting considerable interest among biomedical researchers because of their unrivaled ability to regenerate entire organs. They share the body plan of tetrapod vertebrates and are unique in their ability to regenerate a broad spectrum of damaged organs throughout life, including limbs, spinal cord, brain, lens, skin, ovary, and heart. Understanding the cellular and genetic mechanisms of Axolotl regeneration could have clinical significance for treating human trauma, disease, congenital malformations, and aging. However, with the recent completion of the Axolotl genome, the Axolotl is poised to impact biology beyond tissue regeneration. Well-developed research paradigms that the Axolotl founded prior to the genomic age, including cytogenetics, embryonic development, and physiology, can now be re-visited to significantly impact biomedical research. To further empower regenerative biology and broadly empower research paradigms where Axolotls provide the best animal model, additional resources are needed.
- B. NIH investments have advanced Axolotl research resources, culminating recently with the first genome assembly. In 2002, NIH provided funding to initiate the Salamander Genome Project (SGP). Since that time, continuous NIH NCCR/ORIP support enabled the SGP to develop genomic and bioinformatic resources for the Axolotl community, including the first chromosome level assembly of the Axolotl genome, the largest genome (32 GB) ever assembled. Since 2015, annual, total cost NIH support for Axolotl-related research projects has tripled, with support coming from a variety of NIH institutes (NIAMS, NEI, NICHD, NHLBI, NIDDK, NIGMS, NINDS, OD, and NCI). Importantly, the Axolotl community has a growing number of young investigators that are supported by K99's, T32's, R15's, R01's and Career Development grants. An NIH P40 Resource Center grant supports the maintenance and distribution of Axolotls by the Ambystoma Genetic Stock Center. Many decades of breeding have yielded relatively homogeneous genetic stocks to meet the needs of a research community. NIH support is catalyzing advances in biomedical research through its support of the Axolotl and the AGSC, increasing the likelihood that investigators will use Axolotl models in the future.
- C. There is broad consensus that essential resources are needed to better enable research efforts for a growing Axolotl community. PI's from the Axolotl community met with other salamander PI's from around the world at the "Salamander Models of Biological Research Meeting in 2018 and 2019. The 2018 in Vienna, Austria was an invitation only PI meeting (N = 35) designed to provide a forum for building consensus about general needs for researchers working with salamanders. The 2019 meeting (N=90) brought together PI's and their students and postdocs in a more scientific meeting format. After this meeting, PI's again met to build consensus about key resource needs to advance salamander models, including the Axolotl which is the primary salamander model. Because of the coronavirus epidemic, the 2020 Salamander Models in Cross-Disciplinary Research Meeting was canceled, and the PI meeting was held virtually. Summaries of meeting outcomes were presented to the community via the Axolotl Newsletter. As a result of these meetings, and feedback that was obtained after an initial draft was submitted to the community for comment, broad consensus was reached for the key research resources detailed below.

Section II: Essential Research Resources Needed by the Axolotl Community

- A. Refinement of the Axolotl genome assembly, including nucleotide error correction, SNP identification, gene annotation, and genome browser annotation. The development of the Axolotl genome assembly presented significant challenges given the large size of the genome, however progressive improvements in

sequencing and assembly approaches (many of which were driven by the community) have provided increasingly accurate assemblies that allow for corresponding increases in the power of studies that use Axolotl. While extremely useful in its current state, the assembly is (like most vertebrate genome assemblies) far from perfect. Between 5 and 6 gigabases of the 32 gigabase genome have yet to be placed on genomic scaffolds and the process of annotating genes and other functional features within this large genome is just beginning. Gap filling via long read sequencing has a strong potential to place remaining unscaffolded sequence and will allow unscaffolded regulatory elements and genes to be placed in their appropriate genomic context, which is critical for inferring their function and ultimately translating analyses to humans or other animal models. Identification of segregating SNP and structural polymorphisms within and among Axolotl populations will become increasingly necessary as genome resources allow for more precise analyses. Polymorphisms within genes may contribute to informative phenotypic variation or impact the efficiency of tools that rely on sequence homology (probes, morpholinos, cas9 guide RNAs, homologous repair constructs). Re-sequencing of two individuals has identified a number of SNPs, but sampling of several more individuals will be necessary to characterize the majority of common polymorphisms and more in-depth studies will likely be necessary to identify and genotype more complex structural polymorphisms. Construction of an accessible database of variant sites and integration with the genome browser will aid in studies that use these polymorphism data and integrating these with efforts to characterize epigenetic and noncoding regulatory sequences (Section D). The effective community use of improved assemblies, tracks and annotations will be facilitated by the scheduled release of assembly freezes, regular coordinated updates of annotation/variant tracks and the integration of sequence resources with other experimental data sources (Section G).

- B. Readily available Axolotl stocks, including wildtype, transgenics, and mutants. The Ambystoma Genetic Stock Center (AGSC) is the primary distributor of Axolotls to labs in the US and abroad. The AGSC is funded as an NIH P40 Animal Resource Center through 2025, ensuring the availability of some but not all Axolotl stocks to biomedical research labs into the future. Approximately 25-30 transgenic and knock-out Axolotl lines have been developed in the past few years, largely by Elly Tanaka and her students, and with advances in genome-editing tools like CRISPR-CAS9, these numbers are likely to increase greatly over the next 5 years. The community needs to develop a resource sharing and shipping plan that imports prioritized transgenic and knock-out Axolotls generated by individual labs to the AGSC so that they can be distributed to more broadly enable community efforts. Plans to increase AGSC capacity for living stock management should be prioritized along with the development of sperm cryopreservation to more efficiently manage stocks (Section F).
- C. Axolotl organ, tissue, and cell atlases. Many more developed animal models have benefitted from a gene expression atlas that is searchable by researchers and enables quick assessment of where and when a gene is expressed. For example, researchers using mice have had the GenePaint resource (<https://gp3.mpg.de>) available since 2004, and organ-centric resources, such as the Allen Brain Atlas (<https://portal.brain-map.org>) have also been developed. These sites are searchable by gene and retrieve microscopy images from *in situ* hybridization to that gene's transcript across several stages of mouse embryogenesis or brain development. The Gallus Expression In Situ Hybridization Atlas (GEISHA) serves as a gene expression database for the chicken research community as well as a link to transgenic stock resources (<http://geisha.arizona.edu/geisha/>). Flyatlas (<http://flyatlas.org/atlas.cgi>) serves a similar function for the Drosophila community, but is more extensive because it collates data from many different gene expression initiatives. These kinds of features are inclusive of the Xenbase (<http://www.xenbase.org/anatomy/atlas.do>) and Zfin (<https://zfin.org/action/expression/search>) databases, which serve the *Xenopus* and zebrafish research communities, respectively. This type of resource, if developed for Axolotl, will be valuable to any researcher in navigating decisions about which genes to pursue following a variety of experimentation; the atlas should include *in situ* hybridization, RNA-Seq, and protein expression (antibody, mass spec) results as they become available, and it will need to be continually curated to stay up-to-date. Ultimately, the needs for most researchers will include information at the single-cell level, and the atlas should include this information as it becomes available. An important function of this kind of resource will be to provide reliable markers for specific cell types in Axolotls, which is critical for assigning cell types and defining cell states in future analyses. For all of these features, new information should be curated agnostically with respect to possible research use; while the initial data may

mainly be contributed by regeneration and development studies, any of the other uses for Axolotl as outlined here will also be included.

- D. Validated antibodies, probes, and pharmaceuticals to reproducibly label, manipulate and characterize genes, macromolecules and cells. Relatively few Axolotl-specific molecular probes have been developed. There is a need to compile information within the community about the availability, specificity, and protocols of use for existing antibodies, nucleic-acid probes/constructs, and pharmaceuticals that have been validated in the Axolotl. New resources of these kinds are needed to probe Axolotl biology with rigor and reproducibility. Funding to enable public-private partnerships could speed the development of Axolotl-specific antibodies and molecular probes. Chemical screens that have proven effective in other aquatic models should be pursued to identify pharmaceutical tools and mechanisms underlying Axolotl biological processes.
- E. Genome-wide characterization of epigenetic marks and non-coding regulatory DNA. The characterization of functionally relevant (noncoding) sequences will be critical to understanding gene regulatory logic in the large salamander genome. Multiple existing and emerging approaches are capable of characterizing chromatin compaction or modifications to DNA and histones that regulate fundamental chromatin states. More precise reconstruction of gene regulatory mechanisms will require the development of validated antibodies (Section D, G) or other affinity-capture methods (e.g. endogenous tags) that will enable precise localization of binding sites within tissues and individual cells.
- F. Develop novel, diploid Axolotl cell lines, including pluripotent stem cell lines. Cell lines are generally useful to advance multiple avenues of research, including the mapping of biochemical pathways, characterization of gene functions, genetic and chemical screening, and epigenetic profiling. Additionally, nuclear transfer, which was pioneered many decades ago in the Axolotl, could be used to re-derive mutants and transgenics if methods were developed to create and cryopreserve cell lines. Development of Axolotl cell lines has proven difficult; there is only one available cell line created by David Gardner and Sue Bryant many years ago and it is aneuploid. Support is critically needed to identify best methods and practices for creating Axolotl cell lines.
- G. Methods and tools for sperm cryopreservation. There are no current methods available to cryopreserve Axolotl germplasm for the purpose of managing, banking and re-driving lines. In 2020, Dr. Terrence Tiersch (Aquatic Germplasm and Genetic Resources Center, Louisiana State University) was awarded a NIH Grant (R24OD028443) to develop a method to cryopreserve Axolotl sperm and work with AGSC staff to perform the method and develop capacity to become a repository. If a reproducible method can be identified, it will be important to provide training and outreach to individual investigators and also develop a comprehensive plan to manage Axolotl frozen stocks at national and international levels.
- H. Online databases that integrate various types of data, including genomic, transcriptomic, proteomic, imaging, and phenotypic data. With increasing access to genome resources, methods for genetic manipulation, and highly specific molecular probes, the community anticipates rapid growth in the volume and information content of datasets being generated in the Axolotl system. The development of robust, user-friendly databases, like those that support *Xenopus* (Xenbase) and Zebrafish (Zfin), are needed to integrate various data sources essential to the effective communication and sharing of results within the community, and in translating this information to other animal models. Many of these tracks (homology, gene expression, epigenetics) can be integrated directly into a centralized genome browser, and integration of other data sources can be achieved through the development of relational databases that allow traversal of gene-centric information across studies, and critically cross-referencing these to uniformly defined Research Resource Identifiers (RRIDs). Prototype Axolotl databases exist (Sal-Site, Axolotl-omics) but are maintained by sweat equity. Dedicated funding is needed to sustain, grow and integrate existing database resources.
- I. New Axolotl models of human disease and congenital disorders. The importance of Axolotl as a developmental model is well-established beyond tissue regeneration and thus Axolotls are poised to make genetic discoveries of developmental processes and provide new disease models. The Axolotl genome is not duplicated, and therefore the genes and proteins that regulate embryogenesis can be cross-referenced

directly to mammalian models. It is crucial to leverage and create the resources necessary for developmental biologists to identify the mechanisms that control early embryogenesis and which are likely re-deployed later in life to accomplish regeneration. Additionally, with advances in genome editing approaches and genomic information, many new genetic lines will be developed as models of human disease. These, along with the cloning of existing mutants, make it urgent to develop strategies to manage an explosion of new Axolotl lines over the next five years (Section B). Large salamander chromosomes, which yielded initial insights about mitosis over 100 years ago, can now serve as models to visualize transcription within the context of chromatin three-dimensional structures. Extracts from large Axolotl oocytes can be easily harvested to identify factors that re-program mammalian cancer cells to avert tumorigenesis. The robust Axolotl innate immune response can now be studied beyond its importance in tissue regeneration to reveal anti-microbial and anti-viral proteins. As a final example, the Axolotl's paedomorphic mode of development provides a unique opportunity to investigate the genetic basis of central hypothyroidism, which is poorly known in human thyroid hormone disease, as well as aging.

- J. Training opportunities in Axolotl husbandry, experimental procedures, and bioinformatics. Workshops that provide hands-on training are needed to introduce new investigators and university animal care staff to methods of axolotl husbandry. Additionally, experimental procedures that are routinely performed in axolotl laboratories, including amputation surgeries and embryo microinjection, can better ensure experimental reproducibility and advance community research efforts. Finally, the new genome assembly will likely motivate a growing number of studies that require expertise in the analysis of genome-wide datasets that will require basic knowledge of bioinformatic tools, and also custom solutions to working with the large axolotl genome. This need could be met by a workshop modelled after, or integrated with, the Xenopus Bioinformatics Workshop at the Marine Biological Laboratory, wherein leading bioinformaticians work closely with graduate students and post-docs to analyze real datasets.
- K. Resources to advance Axolotl educational and outreach opportunities. Axolotls are charismatic animals that provide unparalleled opportunities to introduce students to biological research. Many leading scientists today had an early axolotl laboratory experience that motivated a career in biological or biomedical research. With a simple dissecting microscope, the entirety of vertebrate embryogenesis can be observed through the lens of axolotl embryos. Just having a charismatic axolotl in the classroom provides an entry point to discuss neoteny and aging, regeneration, and DNA/genome size. High school students and undergraduates can perform simple surgeries or apply chemicals to investigate regeneration and development. Support for axolotl-based educational activities is needed to attract top students into biological research.
- L. Diagnostic, preventive, and treatment methods to mitigate emerging or potential pathogens of Axolotl facilities. Much has been learned over the last 20 years about microorganisms that are pathogenic to amphibians. Most notably, the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) has been identified as the primary causal pathogen in global anuran declines. A related species, *B. salamandrivorans*, now poses a grave risk to salamander populations around the world. The recent discovery that AGSC Axolotls carry a non-virulent strain of *Bd* provides a community, wake-up call. There is urgent need to develop methods for pathogen monitoring and mitigation, and to more generally investigate Axolotl immunology and disease.

Section III: Conclusions

The Axolotl is the primary salamander model used by NIH-funded investigators. The recent completion of the Axolotl genome, coupled with growth and coalition of the Axolotl community within the larger group of salamander researchers around the world, has revealed new research opportunities and challenges. While incremental NIH investments over the last 18 years were pivotal in establishing the Axolotl model in the genomics age, essential resources are needed to elevate Axolotl research to the level of established animal models and foster the efforts of a growing community of young investigators. This White Paper articulates essential needs of the Axolotl Community.

Section IV: Appendices

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B. Report from the 1st Salamander Models in Cross-Disciplinary Biological Research

The first “Salamander Models in Cross-Disciplinary Biological Research” meeting was held this July 2018 at the Research Institute of Molecular Pathology (IMP) in Vienna, Austria. The meeting organizers included the Ambystoma Stock Center director Randal Voss, along with Jessica Whited (Harvard), Karen Escheverí (Woods Hole MBL) and host Elly Tanaka (IMP, Vienna). This was a gathering of principal investigators working on various aspects of salamander regeneration, development, genetics, and genomics. The major goals of the meeting included reviewing recent advances in research tools, along with nuanced tips for employing them, while also establishing broad consensus for needed research resources for salamander models. Another objective was to lay the groundwork for organizing future salamander meetings intended for the broader community, including postdoctoral and pre-doctoral trainees. The next scheduled meeting will be at Northeastern University in Boston MA on July 22-25th, and will include research symposia and poster sessions.

Participants discussed recent advances in genome sequencing and assembly and the need to make further refinements of a newly presented Axolotl whole-chromosome level assembly. The need for additional transgenic lines and approaches was discussed. A wide range of transgenic and CRISPR edited lines are becoming available, especially in the Axolotl. Discussions focused on prioritizing existing stocks and their dissemination through the AGSC (primarily in North America), Max Planck Dresden and MPI (in Europe). The need to pursue financial resources to enable more extensive repository functions for salamanders was also addressed.

The real value of this PI-focused meeting was to allow researchers to champion the resources they found most pressing for the field. Several additional resources were identified in addition to continued improvements to genome annotations and the temporal control of gene expression. These included detailed histological atlases of regeneration, master lists of validated antibodies, and more stable cell lines for in vitro experiments. One of the most obvious resources needed was the continued communication between labs with subsequent salamander research conferences that should continue to strengthen this growing research community.

Several research communities have benefitted from the intentional development and promotion of particular animal models. One-stop repositories of information such as Flybase, Wormbase, ZFinBase and Xenbase have all expanded the research capabilities of participating labs. While many of these labs focus on Developmental Biology, the reach of these animal model communities includes studies of neuroscience, evolutionary biology, physiology, and ecology. Emulating this deliberate approach will provide vital cohesion and an undoubted boon to investigators studying salamander regeneration and development.

C. Report from the 2nd Salamander Models in Cross-Disciplinary Biological Research

The second annual Salamanders in Cross Disciplinary Biological Research was held in Boston on July 23-25th, 2019. The meeting was built upon the successful first meeting held in Vienna, Austria in 2018. The unique aspect of the second meeting was that it was the first conference to include trainees including undergraduates, graduate students, and postdoctoral researchers. Held on the campus of Northeastern University in the heart of Boston, the was co-organized by Catherine McCusker from University of Massachusetts Boston, Jessica Whited from Harvard University, Karen Echeverri from the Marine Biological Laboratory, and James Monaghan from Northeastern University.

The conference was well attended with 88 attendees from 11 countries, highlighting the true international nature of salamander research. The salamander species represented included the Axolotl, Eastern newt, Spanish ribbed newt, spotted salamander, and even salamander-like reptiles made an appearance. Topics ranged from chromosomal organization, genome assembly, lampbrush chromosomes, gastrulation, epigenetics, DNA damage, endosymbiosis, and regeneration of spinal cord, brain, retina, lens, heart, muscle, joint, tail, limb. The meeting included talks from experienced principal investigators, but importantly, the majority of talks were presented by trainees.

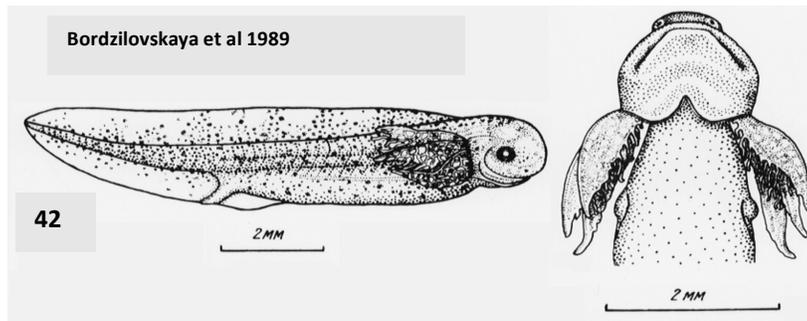
After the meeting, PI's met to discuss needs of the overall salamander community as well as needs of specific salamander models. PI's identified the need to accumulate information about Axolotl transgenic and knock-out lines that could be brought into resource centers like the Ambystoma Stock Center for distribution. Also, the PI's thought it was essential to generate a list of needs and research resources that could be presented to agencies for consideration of targeted funding. Other needs and resources were identified, including the need to develop cryopreservation methods for salamander sperm, refined genome assemblies, compiled lists and further development of antibodies, molecular probes and pharmaceuticals, and curation of genomic and bioinformatic information for salamander models, perhaps in a common database.

Overall, the meeting was highly interactive and built comradery across the broad salamander research community. The next meeting is scheduled to take place in Dresden, Germany in 2021 and has led to an online webinar called the "Salamander League" to occur monthly for the community. Support was provided by an NIH R13 conference grant, Developmental Systems Hybridoma Bank, Zeiss Microscopy, Iwaki Aquatics, and The Society for Developmental Biology.

D. Sample cover of the Axolotl Newsletter

Axolotl

Newsletter of the
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Director's Note – Randal Voss

I hope everyone in the community is healthy and safe, the last few months have certainly been trying. The University of Kentucky sent undergraduates home for the semester in March, including several of our student Axolotl care workers. Soon after this, all but essential research was halted. Care for the AGSC Axolotl population was thankfully deemed essential, and with a reduced workforce, we all pitched in to accomplish Axolotl care. I acknowledge our dedicated student workers (Logan Dortsch, Arabella Jackson, Elise Maul, and Micaiah McNabb) and staff (Chris Muzinic, Laura Muzinic, and Grace Zimmerman) for all of their efforts during the pandemic. Thankfully, labs at UK are starting to open up again; we are hopeful that everyone in the community