Journal of Visualized Experiments

Generation of Chimeric Axolotls with Mutant Haploid Limbs Through Embryonic Grafting

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60156R1
Full Title:	Generation of Chimeric Axolotls with Mutant Haploid Limbs Through Embryonic Grafting
Keywords:	Axolotl, haploid, tissue grafting, transplantation, regeneration, limb, limb bud, chimera, CRISPR, Cas9, multiplex mutagenesis
Corresponding Author:	Lucas Sanor UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	lucas.sanor@yale.edu
Order of Authors:	Lucas D Sanor
	G Parker Flowers
	Craig M Crews
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	New Haven, Connecticut, USA

Cover Letter

Craig M. Crews, Ph.D.
Kline Biology Tower 400
P. O. Box 208103
New Haven, CT 06520-8103
Phone (203) 432-9364
Fax (203) 432-6161
email: craig.crews@yale.edu
www.yale.edu/crews

YALE UNIVERSITY
DEPARTMENT OF MOLECULAR, CELLULAR & DEVELOPMENTAL BIOLOGY
DEPARTMENT OF CHEMISTRY
DEPARTMENT OF PHARMACOLOGY

May 30, 2019

Dear Dr. Vineeta Bajaj,

We appreciate your great interest and suggestions for our manuscript "Generation of chimeric axolotls with mutant haploid limbs through embryonic grafting." We greatly appreciate that the reviewers note the detail and rigor of the protocol and that it will be a great resource to the field. The described method provides a way to reduce the time and space required for functional screening of genes implicated in limb regeneration in the axolotl by generating chimeric axolotls with mutant haploid limbs, and we are happy that the reviewers universally acknowledge the great value of this method. We thank them for their input, as it has led to a number of substantial improvements in the manuscript.

The current submission contains a number of changes to address the reviewers' comments. We have included a flow chart and additional detail to more clearly convey the parallel steps of the protocol. We have included more text to describe the history of research with haploid salamanders, the advantages of conducting mutagenesis in haploids over diploids, and potential limitations of this technique. We have clarified several figures by providing bright-field images and outlined areas of interest within images. We have included additional data and images to report the morphological differences between haploid and diploid limbs and to demonstrate the complete regeneration of haploid limbs. We have added data to report the success rates of egg activation. We have successfully added a missing figure (which was errantly omitted previously), changed the formatting to adhere to JoVE's editorial standards, and uploaded several videos demonstrating different steps of the protocol. We believe that these changes have addressed the reviewers' concerns and markedly increased the quality of the manuscript.

As we have discussed over the phone with different editors and with you via email, we have submitted a concurrent manuscript using the techniques detailed in this protocol to research candidate genes in regeneration. This manuscript is currently under review. This additional manuscript contains a more detailed characterization of the quality and rate of regeneration of haploid limbs, including a figure. As reviewers 3 and 4 asked us to elaborate upon these topics, we have provided additional data and images. These data and figures are not the same as those in the additional manuscript to avoid any potential conflicts.

As mentioned before, because of upcoming construction and relocation which will shut down our axolotl facility and the associated lab space, this concurrent submission was required if we are to film the necessary footage for this video protocol while the lab space is accessible. If you choose to publish this work, we request that the filming be scheduled in a timely fashion, even as we temporarily withhold its publication to coincide with the concurrent manuscript's acceptance and publication.

Thank you for your consideration, and we look forward to your decision.

Craig M. Crews, Ph.D.
Lewis B. Cullman Professor of Molecular, Cellular and Developmental Biology
Professor of Chemistry
Professor of Pharmacology
Executive Director, Yale Center for Molecular Discovery

TITLE:

Generation of Chimeric Axolotls with Mutant Haploid Limbs Through Embryonic Grafting

AUTHORS AND AFFILIATIONS:

Lucas D. Sanor[1], G. Park Flowers[1], Craig M. Crews[1]

[1]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, USA

Email addresses of co-authors:

Lucas D. Sanor (lucas.sanor@yale.edu)

G. Park Flowers (grant.flowers@yale.edu)

Corresponding author:

Craig M. Crews (craig.crews@yale.edu)

KEYWORDS:

axolotl, haploid, tissue grafting, transplantation, regeneration, limb, limb bud, chimera, CRISPR, Cas9, multiplex mutagenesis

SUMMARY:

This goal of this protocol is to produce chimeric axolotls with haploid forelimbs derived from Cas9-mutagenized donor tissue using embryonic tissue grafting techniques.

ABSTRACT:

A growing set of genetic techniques and resources enable researchers to probe the molecular origins of the ability of some species of salamanders, such as axolotls, to regenerate entire limbs as adults. Here, we outline techniques used to generate chimeric axolotls with Cas9-mutagenized haploid forelimbs that can be used for exploring gene function and the fidelity of limb regeneration. We combine several embryological and genetic techniques, including haploid generation via in vitro activation, CRISPR/Cas9 mutagenesis, and tissue grafting into one protocol to produce a unique system for haploid genetic screening in a model organism of regeneration. This strategy reduces the number of animals, space, and time required for the functional analysis of genes in limb regeneration. This also permits the investigation of regeneration-specific functions of genes that may be required for other essential processes, such as organogenesis, tissue morphogenesis, and other essential embryonic processes. The method described here is a unique platform for conducting haploid genetic screening in a vertebrate model system.

INTRODUCTION:

Historically, embryonic tissue grafting in amphibians has been an important technique for exploring fundamental mechanisms of developmental biology and regeneration. The axolotl, a species of salamander, possesses an impressive ability to regenerate tissues and complex structures such as limbs and organs after injury or amputation. Similarly impressively, they can receive, without rejection, tissue grafts from other individuals at embryonic, juvenile, and adult

stages[1–3]. Regions of embryos that produce whole structures such as limbs, tails, eyes, and heads, and more specific tissues, such as neuroectoderm and somites, can be grafted between embryos to produce chimeric animals[1,2,4–6]. For nearly a century, studies of such chimeric animals have provided crucial insights into regeneration, tissue differentiation, size control, and patterning[1,7,8].

In the last the decade, numerous transcriptional studies of regenerating tissues have produced insights into the genetic programs underlying salamander regeneration[9–13]. These studies have added to an expanding list of candidate genes that, to date, are largely uncharacterized in the context of regeneration. Targeted mutagenesis techniques, such as CRISPR/Cas, now permit the investigation of such genes, and such genetic approaches are greatly facilitated by the recent sequencing and assembly of the large axolotl genome[14–16].

We sought to develop techniques that coupled classic developmental biology with new genetic technology for the purpose of dissecting the mechanisms of regeneration. Methods for generating haploid embryos of axolotls and other salamanders have been established for decades[17]. While these techniques have long been noted to be advantages of salamanders as genetic model organisms[18], few subsequent genetic studies have incorporated haploid animals. We use in vitro activation in the axolotl to produce haploid embryos that serve as tissue donors for grafting[19]. Using embryos carrying fluorescent genetic markers, we have devised reliable methods for generating limbs derived almost entirely from donor tissues (**Figure 1A**). By combining these two techniques, we have bypassed the late embryonic lethality associated with haploidy, allowing for the production of fully developed, grafted haploid limbs (**Figure 1B**, **Figure 1B'**, and **Figure 2**).

We sought to develop techniques that coupled classic developmental biology with new genetic technology for the purpose of dissecting the mechanisms of regeneration. Using embryos carrying fluorescent genetic markers, we have devised reliable methods for generating limbs derived almost entirely from donor tissues (**Figure 1A**). We use in vitro activation in the axolotl to produce haploid embryos that serve as tissue donors for grafting[19]. By combining these two techniques, we have bypassed the late embryonic lethality associated with haploidy, allowing for the production of fully developed, grafted haploid limbs (**Figure 1B**, **Figure 1B'**, and **Figure 2**).

By conducting CRISPR/Cas-mediated mutagenesis in haploid embryos prior to grafting to create chimeric axolotls with mutant haploid limbs, we may investigate gene function specifically within the context of limb development and regeneration. This allows the rescue of limbs from potentially embryonic-lethal mutant phenotypes. While CRISPR/Cas microinjection can generate animals that are highly mutant, such animals are typically highly mosaic, with some degree of retention of wildtype alleles and a variety of distinct mutations at targeted sites[14,20]. CRISPR-based mutagenesis in haploid cells increases the penetrance of single allele loss-of-function mutations, as they cannot be masked by retained wildtype alleles. For this reason, CRISPR-based screening in haploid cell lines is increasingly used to investigate the genetic basis of many cellular processes[21–23]. By combining CRISPR-based lineage tracing with our haploid limb bud grafting protocols, the approach described here can serve as a platform for haploid genetic screens in living animals[20].

PROTOCOL:

Experimental procedures used in this protocol were approved by the Yale University Institutional Animal Care and Use Committee (IACUC, 2017–10557) and were in accordance with all federal policies and guidelines governing the use of vertebrate animals. All animal experiments were carried out on *Ambystoma mexicanum* (axolotls) in facilities at Yale University.

1. Diploid embryo generation

1.1. Obtain GFP+ diploid embryos to serve as graft hosts through natural mating using one or two *gfp* parents[24].

1.2. Collect freshly laid diploid eggs and place them in a metal sieve.

1.3. Rinse the eggs thoroughly with 40% Holtfreter's solution (20 mM NaCl, 0.2 mM KCl, 0.8 mM $NaHCO_3$, 0.2 mM $CaCl_2$, 4 mM $MgSO_4$, pH to 7.4).

1.4. Place the eggs in fresh 40% Holtfreter's solution and store at 12 °C.

NOTE: Diploid embryos should always be obtained before moving on to haploid embryo generation.

2. Haploid embryo generation

2.1. Female gamete donor preparation

2.1.1. 48 hours before conducting in vitro activation, anesthetize a sexually mature white or white/RFP female axolotl by immersion in 1 g/L HEPES-buffered MS-222 in 40% Holtfreter's solution[25].

2.1.2. Ensure that the animal is fully anesthetized after approximately 30 min of immersion by firmly pinching its tail between the thumb and forefinger. Fully anesthetized animals will not physically respond to any pinch.

2.1.3. Prepare a solution of human chorionic gonadotropin (HCG) containing 10,000 U/mL in sterile saline.

2.1.4. Using a 30 G insulin syringe, inject 1.5 CC of HCG (1,500 units) into the musculature dorsal to the hind limb of the anesthetized female at 45° angle to the midline to avoid contact with the spinal cord.

2.1.5. Return the female to fresh 40% Holtfreter's solution and place in an 8–12 °C refrigerator.

2.1.6. After 48 h, return the female to room temperature. Place a few stones or plastic plants in her container as surfaces for egg laying.

NOTE: Females injected with HCG will often deposit empty jelly cases for several hours before laying eggs.

2.1.7. Remove the stones or plastic plants after the female has begun consistently laying eggs.

2.1.8. Allow the female to sit in the empty tank for 30 min to 1 h.

NOTE: Withholding materials for the animal to lay eggs on will allow tighter control of oocyte collection.

2.2. Male gamete collection

2.2.1. While the female's egg laying is stalled, anesthetize a sexually mature GFP+ male axolotl to serve as a sperm donor, as in step 2.1.1.

2.2.2. Place the fully anesthetized male on his back on damp paper towels under a dissecting microscope.

2.2.3. If the experimenter is righthanded, position the tip of a P1000 pipette at the base of the cloaca with the right hand.

2.2.4. Place the left forefinger and thumb of the left hand 2–3 cm rostral to the pelvis. Gently squeeze the animal while moving the fingers towards the hind legs to flush out spermic urine samples.

2.2.5. Collect each that is flushed out of the cloaca into an individual microcentrifuge tube. Repeat this process to collect 6 to 10 samples.

2.2.6. Pipette 5.0 µL from each sample onto a petri dish to inspect the quality of the sperm using an inverted microscope.

NOTE: Concentrated sperm samples are a milky white color, typically range from 5 to 20 µL, and are usually retrieved after several, higher volume spermic urine samples are extracted. Sperm will collect at the bottom of the tubes in higher volume samples when left undisturbed. Concentrated samples of healthy sperm are highly motile and lose activity as their concentration decreases. Healthy males can produce up to 50 µL of concentrated sperm.

2.3. Female gamete collection

2.3.1. After obtaining and confirming a healthy sperm sample, anesthetize the HCG-injected female axolotl as in step 2.1.1.

2.3.2. Place the fully anesthetized female on damp paper towels on her back.

2.3.3. Extract unfertilized eggs from the female using a hand motion similar to that in step 2.2.4.

2.3.4. Collect the eggs using wet forceps and transfer them to a 10 cm petri dish. Treat eggs with irradiated sperm within 15 min of collection.

2.4. Male gamete preparation and in vitro activation

2.4.1. Use a P10 or P20 pipette to pipette the sperm up and down, breaking apart the clumps to form a homogenous suspension.

2.4.2. Approximate the percentage of motile sperm by placing a 0.5 µL drop of undiluted sperm suspension on a petri dish lid or glass slide and examining with an inverted microscope.

2.4.3. Add 9.5 µL of 0.1x Marc's modified Ringer's solution (MMR; **Table of Materials**) to this sample to make a 20x sperm dilution. Gently pipette up and down to mix.

2.4.4. With an inverted microscope, count the sperm in three 1.0 µL drops of the 20x diluted aliquot on a petri dish cover or hemocytometer to estimate the sperm concentration of the undiluted suspension.

2.4.5. Prepare the sperm for irradiation by diluting an aliquot of the original sperm sample to about 80,000 motile cells/mL in sterile 0.1x MMR.

2.4.6. Count the number of eggs obtained within the past 15 min. Add 0.5 µL of the freshly diluted sperm from step 2.4.5 per egg to a petri dish. Use the pipette tip to spread this suspension into a 1 mm thick layer.

2.4.7. Using a plastic lift, place the sample 4 cm from the bulbs of a 254 nm crosslinker. Genetically inactivate the sperm by irradiating the sample with 800,000 uJ/mm^2.

2.4.8. Using a P10 pipette, pipette the suspension onto the unfertilized eggs, coating each egg with 0.25−0.5 µL of irradiated sperm. Allow the eggs to sit at room temperature for 30 min.

2.4.9. After 30 min, flood the eggs with 0.1x MMR. Immediately place the eggs in an 8−10 °C incubator for injections the next day or at 18 °C for injections within 7 hours post activation (hpa).

2.4.10. Dejelly haploids using sharp forceps 30 min after hydration. Immediately place the eggs in an 8−10 °C incubator for injections the next day or at 18 °C for injections the same day.

3. Haploid mutagenesis and maintenance

3.1. CRISPR/Cas9 microinjections

3.1.1. Design sgRNAs using CRISPRscan and synthesize[26,27].

3.1.2. For multiplex mutagenesis, prepare a stock of 5 sgRNAs (10 ng/µL for each sgRNA) and Cas9 protein (1 µg/µL). Prepare a 100-fold dilution of this stock (0.1ng/µL per sgRNA, 10 ng/µL Cas9) for injection. For single gene, high mutation frequency mutagenesis, follow the protocol outlined previously[28].

3.1.3. Inject haploid embryos at the single cell stage 7 hpa if stored at 18 °C or inject embryos the next day at the 2−8 cell stage if they are stored at 8−10 °C.

3.1.4. Transfer the embryos to 1.0x MMR with 20% polysucrose 400.

3.1.5. If single cell, inject each embryo with a 5 nL drop of the injection solution (approximately ¼ radius of the egg) containing a total mass of 0.5 pg/sgRNA and 50 pg Cas9 protein. If multicellular, distribute this mass by injecting smaller volumes into multiple cells.

3.1.6. Allow the embryos to heal in the polysucrose 400 for a minimum of 4 h and a maximum of 18 h at 18 °C.

3.2. Haploid embryo housing

3.2.1. Transfer the embryos to 0.1x MMR with antibiotic-antimycotic.

3.2.2. House each embryo in an individual well of a 24-well plate, as some will die or develop abnormally. Maintain at 16−18 °C. Lower temperatures can be used to prolong development, if necessary.

3.2.3. Replace the media with fresh 0.1x MMR with antibiotic-antimycotic every other day.

4. Diploid host embryo preparation

4.1. Maintain embryos in the jelly coating at 12 to 16 °C until they reach stage 22 to 26.

4.2. Collect the embryos that are ready for grafting, place them within a sieve (4 mm mesh size), and gently rinse them with 40% Holtfreter's solution.

4.3. Transfer the embryos into filter-sterilized 0.1x MMR containing 1.5% bleach for up to 2 min. Completely submerge the embryos in the bleach solution and swirl them gently to ensure that the jelly coating makes full contact with the bleach solution to kill the microbes present on the embryos.

4.4. After 2 min, dilute the bleach solution containing the embryos with an equal volume of filter-

sterilized 0.1x MMR.

4.5. Gently pour the embryos into a bleach-sanitized sieve (4 mm mesh size) and rinse the embryos five times with filter-sterilized 0.1x MMR.

4.6. Place the embryos into sterile 10 cm petri dishes with 0.1x MMR with antibiotics for dejellying (penicillin 100 units/mL, streptomycin 100 µg/µL, 0.25 µg/mL, gentamicin 25 µg/mL).

4.7. Under a fluorescent stereomicroscope, remove the jelly coats and vitelline membranes from GFP+ embryos using sharp forceps (tip dimensions 0.05 x 0.01 mm).

4.8. Transfer the GFP+ embryos to a new petri dish. Minimize the amount of liquid transferred from the petri dish where they were dejellied.

4.9. Rinse the GFP+ host embryos with sterile 0.1x MMR with antibiotics four to six times in order to remove contaminants.

4.10. Place the clean embryos at 4 °C overnight before grafting.

NOTE: Cooling the embryos makes them more rigid and clean separation of the mesoderm from the endoderm feasible.

5. Haploid-diploid chimera generation

5.1. Surgical dish and media preparation

5.1.1. Prepare sterile surgical operating dishes by pouring autoclaved 2% agarose in 0.1x MMR into sterile 35 mm easy-grip petri dishes. Fill the petri dishes halfway with agarose.

5.1.2. After the agarose cools, use a sterile scalpel to cut a 25 mm long, slanted trough in the agarose to hold the embryos in place.

5.1.3. Fill the dish with sterile surgical media (0.1x MMR with anti-mycoplasma 2.5 µg/mL, amphotericin B 0.25 µg/mL, and ciprofloxacin 10.0 µg/mL) and refrigerate at 4 °C.

5.2. Embryo grafting procedure

5.2.1. Place one healthy haploid donor with one or two stage-matched GFP+ diploid host embryos inside the trough of the prechilled operating dish containing surgical media (**Figure 3**).

NOTE: Perform the procedure on a cooling stage (10 °C or lower) if possible.

5.2.2. Use two ultra-fine, autoclaved forceps (straight tip, tip dimensions 0.05 x 0.02 mm) to remove the ectoderm and mesoderm layers from the host with the limb bud near the center (**Figure 4**).

NOTE: The rectangular tissue graft region encompasses the limb bud and extend from the ninth somite to the posterior half of the gill bulge, about 2 mm, along the anterior posterior axis. Along the dorsoventral axis, the grafted region spans approximately 1.5 mm, including the somites to just beyond the ventral edge of the gill bulge. The grafted region includes all lateral plate mesoderm and the lateral halves of the somites, without disturbing underlying endoderm. See **Figure 4** and the accompanying video for details.

5.2.3. Set aside the host tissue and remove an equivalently sized tissue sheet from the haploid donor using the same methods.

5.2.4. Place the haploid donor tissue sheet onto the corresponding region of the donor embryo.

5.2.5. Secure the tissue by covering it with an autoclaved, rectangular glass shard from a crushed microscope cover glass and gently pressing it into the host embryo body.

5.2.6. Flip the haploid donor embryo onto its other side to harvest the limb bud for the second host embryo. Repeat steps 5.2.2 through 5.2.5.

5.2.7. Carefully remove the remaining excess host tissues and the donor embryo from the dish.

5.2.8. Leave the grafts with the glass shard anchors in place for 60−75 min, checking every 20 min to ensure that the glass has not slipped off.

5.2.9. After the tissue grafts fully adhere, use the forceps to slowly peel off the glass shard anchors.

5.3. Chimera maintenance

5.3.1. Transfer the engrafted embryos to fresh surgical media and maintain them at 8−12 °C overnight to heal. Individually house engrafted embryos in 12 or 24-well plates.

5.3.2. After 36−48 h, transfer the engrafted embryos to sterile 0.1x MMR antibiotic-antimycotic. The strong antibiotics in the surgical media cause toxicity in engrafted embryos after 3 days.

5.3.3. Replace the media with fresh 0.1x MMR and antibiotics every 2−3 days.

5.3.4. Maintain the engrafted embryos at 18 °C until they are able to feed[28].

5.3.5. After 1 to 2 months of development and care, haploid limbs can be scored for purity of the graft using a fluorescent dissection microscope.

NOTE: The presence of non-neural or non-blood host-derived GFP-tissue in limbs is an indicator of impure grafting and is often associated with abnormal limb development. These animals should be excluded from further analysis.

REPRESENTATIVE RESULTS:

Developing haploid embryos can be distinguished from diploid embryos by their 'haploid syndrome' phenotype[29]. At graft-stage, haploid embryos exhibit reduced curvature along the anterior-posterior axis and incomplete enclosure of the yolk plug (**Figure 3A**). A fluorescent microscope can be used to ensure that haploid embryos are free of paternally derived GFP expression (**Figure 3B**).

When grafts are clean, GFP expression should be limited primarily to the brachial plexus, the neural network derived from the host's spinal cord, as seen in **Figure 2**. Punctate GFP expression will also be present in single cells that appear to be sensory neurons and blood-derived host cells, which migrate into the developing limb. When RFP+ donors are used, haploid graft limbs will show universal expression of RFP (**Figure 1B'**). Throughout development, non-mutagenized haploid limbs are significantly shorter than the opposing diploid forelimbs in size-matched chimeric animals (**Figure 1B**, **Figure 1B'**, and **Figure 5**, n = 16 limb pairs, haploid mean = 0.522 cm, SD = ± 0.087 cm, diploid mean = 0.667 cm, SD = ± 0.069 m, paired T-test p-value < 0.0001, mean ratio = 0.784, SD = ± 0.113). Non-mutagenized haploid limbs also fully regenerate (4/4 haploid and 4/4 diploid complete regeneration, **Figure 6**), though they show a slight delay in reaching the digital outgrowth stage compared to diploids (n = 4 haploid, n = 4 diploid; 23 days after amputation: haploids = 3 palette and 1 digital outgrowth stage limbs; diploids = 4 digital outgrowth stage limbs).

Successful grafting requires practice, and consistency will vary depending on the technician's micromanipulation skills and sterile technique. Failed and impure grafts produce a variety of phenotypes, as seen in **Figure 7** and **Figure 8**. In our hands, approximately 38.7% (SD = ± 8.78%) of oocytes develop into normally developed haploid embryos and 11.1% (SD = ± 5.46%) of all haploid-diploid grafts produce viable animals with normally developed, cleanly grafted haploid limbs (**Figure 9**).

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of protocol and an example of a chimeric axolotl. (**A**) Schematic outlining the relative timing of steps taken to obtain diploid host embryos, sperm, and eggs in order to generate haploids and chimeric embryos. (**B**) Composite bright image of a juvenile axolotl produced by embryonic limb bud grafting from an RFP+ haploid embryo to a GFP+ diploid host (**B'**) Overlay of green and red fluorescent images of the same 8 cm juvenile axolotl. The haploid limb is grossly normal, but shorter than the opposing diploid limb. Scale bar = 1 mm.

Figure 2: Fluorescent image of a normally developed and cleanly grafted haploid limb. GFP-haploid limb grafted to a GFP+ diploid host shows GFP expression pattern that appears to be

restricted to spinal nerves innervating the limb (yellow arrow) and individual sensory neurons and blood-derived cells (white arrows). The limb pictured belonged to a 4 cm juvenile. Scale bar = 1 mm.

Figure 3: Comparison of diploid and haploid embryos. (**A**) Light image of diploids (left) and haploids (right). Note the reduced AP-axis curvature and the protruding endoderm of the haploid embryos (white arrows). (**B**) Green fluorescent image of the same embryos. GFP- haploids were generated using eggs from a GFP- female and irradiated sperm from a GFP+. Diploids are GFP+. Embryos pictured are approximately stage 25[30]. Staging series. Scale bar = 1 mm.

Figure 4: Schematic outlining the extent of a haploid limb bud graft. (**A**) Lateral view of a stage 25 haploid embryo. The dotted lines show the approximate area that should be transplanted, relative to the gill bulge and limb bud. (**B**) Transverse schematic of stage 25. The dotted lines show the approximate area and tissue types that should be removed from the host and replaced with the corresponding haploid donor tissue.

Figure 5: Comparison of haploid and diploid limb lengths. (**A**) Scatter plot of the lengths of haploid limbs (pink) and opposing diploid limbs (green) of 16 similarly sized chimeric animals (haploid mean = 0.522 cm, SD = ± 0.087 cm, diploid mean = 0.667 cm, SD = ± 0.069 cm). Limbs were measured from the base of the zeugopod to the junction of digits 2 and 3. (**B**) Scatter plot of the haploid limb length to diploid limb length ratios of the 16 animals (mean ratio = 0.784, SD = ± 0.113 cm). (**C**) Scatter plot of the body lengths (cm) of the 16 measured chimeras (mean animal length = 8.72 cm ± 0.456 cm). Animals were measured from the tip of the snout to the tail tip.

Figure 6: Comparison of regeneration in haploid and diploid limbs. (**A**) Haploid limb pre-amputation. (**B**) The same haploid limb after regeneration. (**C**) Diploid limb pre-amputation. (**D**) The same diploid limb after regeneration. 4/4 haploid limbs and 4/4 diploid limbs regenerated with normal morphology. Black dotted lines indicate the amputation plane. Scale bars = 1 mm. Amputations were performed on size-matched juvenile animals (8.5 cm).

Figure 7: Examples of normal and abnormal development of haploid limbs. (**A**) A haploid limb with grossly normal morphology. (**B-E**) Examples of grafts that failed to support complete development of haploid. (**B**) Oligodactyly. (**C**) More severe oligodactyly. (**D**) No distinct digital structures are present. (**E**) No limb. The limbs pictured belong to similarly sized juveniles (8.0 to 10 cm). Scale bars = 1 mm.

Figure 8: Examples of GFP+ cells in impure haploid limb grafts. (**A,A'**) Morphologically normal haploid graft limb with GFP+ diploid cells in the skin and dermis (white dashed outline) revealed by fluorescent microscopy. (**B,B'**) A grafted abnormal haploid limb with GFP+ diploid cells contributing extensively to the dermis and skin (white outline). (**C,C'**) A grafted abnormal haploid limb in which the epidermis has been replaced with GFP+ diploid cells (white outline). The limbs pictured belong to similarly sized juveniles (8.0 to 10.0 cm). Scale bar = 1 mm.

Figure 9: Success rates of haploid embryo and haploid limb generation. (**A**) Percent of viable haploid embryos generated using in vitro activation. Data were collected from five independent in vitro activation experiments. Green indicates the fraction of eggs that produced stage 25 haploid embryos that could be used for grafting (mean = 38.7%, SD = ± 8.78%). Grey indicates the fraction of eggs that did not show signs of cleavage, were non-viable, or were normal in development. (**B**) Percent of normally developed, cleanly grafted haploid limbs. Purple indicates the fraction of grafts that yielded clean, normally developed haploid limbs (mean = 11.1%, SD = ± 5.46%). Green indicates limbs that developed normally but had contaminating GFP+ host tissues (mean = 11.3%, SD = ± 8.64%). Red indicates graft limbs that did not develop normally (mean = 27.1%, SD = ± 30.3%). Blue indicates all grafted embryos and animals that did not survive to complete limb development (mean = 50.5%, SD = ± 31.2%). Loss of grafted animals was distributed across late embryonic and larval stages**.**

DISCUSSION:

There are a few critical steps in our protocol for generating haploid-diploid chimeras that the operating technician should consider for consistent grafting results.

The most likely reason for haploid generation to fail is due to poor in vitro activation conditions. The proper quantities of motile sperm must be used to activate eggs. To prolong motility, sperm samples should always be maintained at 4 °C. Before applying any sperm sample to eggs, check the viability of the sperm using an inverted microscope. Completely non-motile sperm should never be used, and the concentration should be adjusted to 80,000 motile cells/mL. Too much sperm in the activation step can also prevent normal egg development. Sperm pits, which appear as small, dark indentations on the surface of the egg, are a physical indication that a sperm cell has penetrated the egg and typically appear 20 to 60 min after applying sperm. Eggs with ten or more sperm pits may not activate properly.

Unfertilized eggs can be activated if collected within 15 min after being laid under water, placed in a dry petri dish, and dried thoroughly with paper towels prior to sperm application. Alternatively, as shown in the video, eggs may be squeezed from a hormone-injected female. We find that these “dry” eggs give more consistent results, though we regularly employ both methods.

Successful grafting requires the proper coupling of diploid embryos with appropriately staged haploid limb bud tissue. This protocol contains a number of measures to ensure stage-matching. As natural mating does not always result in egg laying, HCG injection to obtain haploid oocytes should not be performed until the naturally mated female begins to lay diploid eggs. After this injection, the induced female should be housed at reduced temperature (8 to 12 °C), which will reduce the rate of egg laying. Housing the hormone-stimulated female at normal temperatures may result in most of the eggs being laid in a short period of time. The onset of egg laying in a chilled female indicates that the female is ready for anesthesia and oocyte extraction. Oocyte activation must occur within 15 min of oocyte extraction. Because activated haploid embryos will be several days behind the naturally produced diploid embryos in development, we recommend incubating diploid embryos at 12 °C while increasing the relative rate of development of the

haploids by incubating them at 18 °C. Because there will be some variation in the interval between HCG-injection and egg laying of induced females, slight adjustments in the incubation temperatures of either haploids or diploids may be necessary so that the staging of host and donor embryos are paired at the time of grafting. Embryos should be chilled to 4 °C for several hours prior to grafting, and this nearly arrests development. Differing the time of onset of chilling haploid and diploid embryos prior to grafting can enable stage-matching. While haploid embryos differ morphologically from diploids, both haploids and diploids reach stage 21 after the neural folds close and the embryos lie one their sides. Like diploids, stage 25 haploid embryos have prominent gill and pronephric bulges. The large head protrudes at an angle relative to the body in haploids, though this is greatly reduced relative to stage 25 diploid embryos, whose heads protrude from the body at nearly a right angle[30].

Sterile technique is absolutely critical for successful tissue grafting. Sterile conditions should be maintained through the entirety of the experiment from haploid generation through the chimeras' embryonic development. We recommend keeping all parent animals in clean, non-system water conditions during the egg laying period to minimize the amount of contaminating organic material at the start of the procedure. Consistent, daily removal of deceased or dying embryos through the entire process is important for maintaining the health of the other embryos. We recommend individually housing all haploid and chimera embryos to minimize cross contamination.

Embryonic microdissection and tissue grafting skills are acquired through practice. During the grafting process, it is important not to puncture or tear the limb bud itself. Tissues being removed from the host and donor embryos should extend beyond the limb bud, as pictured, to provide a buffering zone. If too small of an area of tissue is grafted, the haploid limbs will be infiltrated by GFP+ diploid skin and other tissues.

One of the limitations of this grafting technique is that the neural tissue and blood in the limbs are derived from the host body. In a few rare cases, we have been able to generate grafted haploid limbs which completely lack all signs of GFP+ expressing nerves. In these cases, we were able to replace enough of the neural tissue in the host animal with that of the donor. However, such extensive grafting is difficult, unreliable, and often produces developmental abnormalities outside of the limb.

Haploidy is embryonic lethal in the axolotl. Similarly, mutations in many genes potentially essential for limb development and regeneration are also early embryonic lethal. Our protocol bypasses the embryonic lethality of haploidy for the production of experimental limbs and could also be used in cases where mutation of a candidate regeneration gene produces an embryonic lethal phenotype. Our limb bud grafting technique provides an advantage for accomplishing this versus previously described methods of limb bud and contributing tissue grafting, as it is performed during early embryonic development and involves the transplantation of complete ectoderm and mesoderm layers[1,2].

Haploid limb bud grafting has been previously described; however, in this earlier study, haploid limb buds were grafted ectopically[31]. Microscopic nuclear analysis of the ectopic limbs derived from these grafts revealed extensive contributions of diploid tissue. While these limbs developed abnormally, they were able to regenerate[31]. Rather than grafting ectopic limb buds, we replace the entire endogenous limb bud with equivalent haploid tissues. Through the use of fluorescent markers, we are able to visually identify haploid limbs that are free of diploid contributions, with the exception of nerve and blood cells, without sacrificing the haploid limb itself. We find that haploid limbs develop and regenerate normally. However, limbs in which GFP+ hosts contribute to tissues other than neural and blood-derived cells often display abnormalities. Thus, when investigating gene function in haploid limbs, those with excessive host contributions must be excluded from analysis before a genotype can be associated with any phenotype observed in mutant haploid limbs.

Recent innovations, like the assembly of the axolotl genome and recent CRISPR/Cas-based insertional methods[15,16,32], have dramatically expanded the genetic malleability of the axolotl. Methods to restrict genetic manipulations temporally and spatially hold great promise, but their current applications are restricted by the resources needed to generate, house, and distribute animal lines. The protocol detailed here accelerates the process by which the consequences of genetic perturbations of genes may be investigated in limb development and regeneration. There has been little characterization of many of the genes found to be upregulated in regenerating limbs, and these genes may be involved in a variety of essential developmental and cellular processes. While we anticipate that many genes required for limb regeneration will also be required for limb development, this method may uncover whether any genes implicated in regeneration have loss-of-function phenotypes that are regeneration-specific. CRISPR/Cas mutagenesis in axolotls typically produces allelic mosaicism that includes retained wildtype alleles, and this mosaicism itself may be regarded as a quantifiable phenotype[20]. Using Next Generation Sequencing of amputated original and regenerated limbs, researchers may quantify whether haploid cells with mutations in a gene of interest are preferentially lost after regeneration. This approach may permit the investigation of regeneration phenotypes produced by perturbation of otherwise essential developmental genes.

Haploid loss-of-function genetic screens facilitate the investigation of the genetic origins of many biological processes without the need to establish biallelic mutant cell lines. By combining haplogenesis, CRISPR/Cas9 mutagenesis, and limb bud grafting, we provide a novel platform for a haploid genetic screen exploring limb development and regeneration in a living animal.

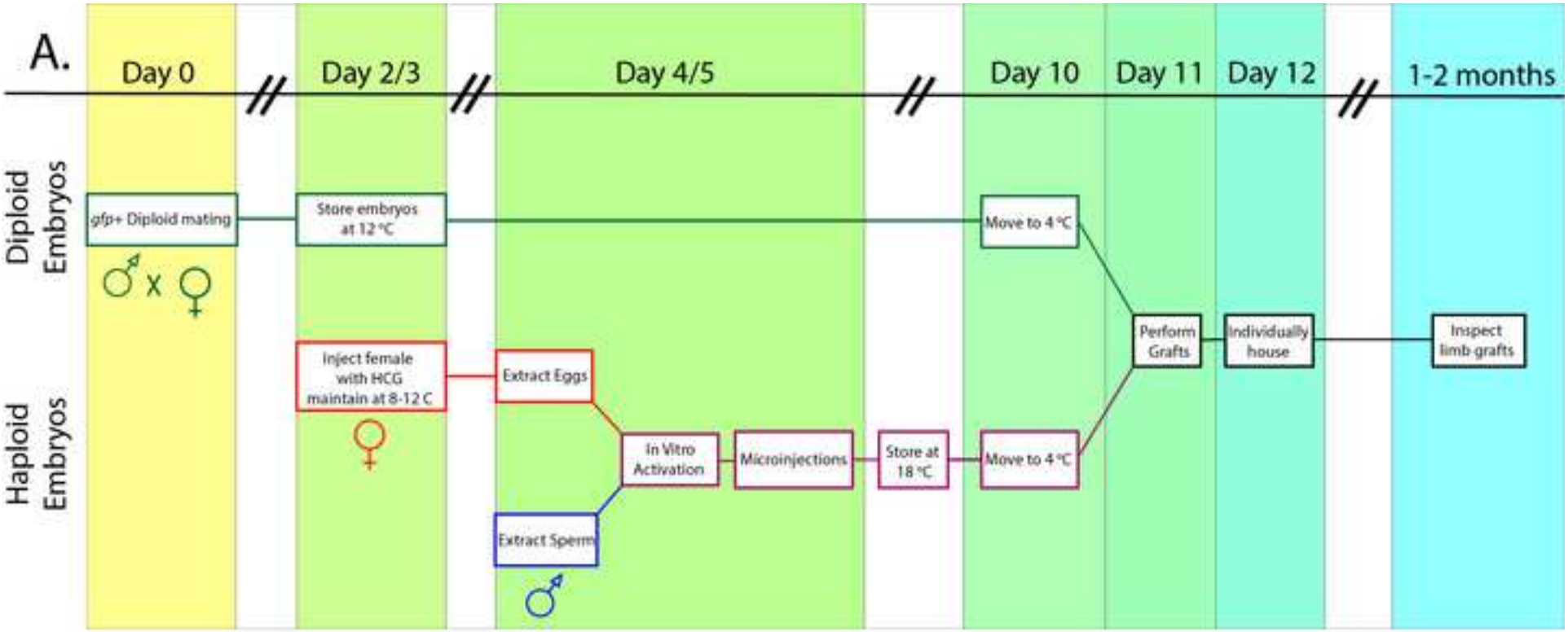
ACKNOWLEDGMENTS:
We would like to thank Katherine Roberts for her care of the axolotl colony. Funding for this work was provided by the Connecticut Innovations Regenerative Medicine Research Fund (15RMA-YALE-09 and 15-RMB-YALE-01) and the Eunice Kennedy Shriver National Institute of Child Health and Human Development (Individual Postdoctoral Fellowship F32HD086942).

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:

1. Kragl, M. et al. Cells keep a memory of their tissue origin during axolotl limb regeneration. *Nature*. **460** (7251), 60–65, doi: 10.1038/nature08152 (2009).
2. Maden, M., Goodwin, B.C. Experiments on developing limb buds of the axolotl Ambystoma mexicanum. *Journal of Embryology and Experimental Morphology*. **57**, 177–187 (1980).
3. McCusker, C.D., Diaz-Castillo, C., Sosnik, J., Q. Phan, A., Gardiner, D.M. Cartilage and bone cells do not participate in skeletal regeneration in Ambystoma mexicanum limbs. *Developmental Biology*. **416** (1), 26–33, doi: 10.1016/j.ydbio.2016.05.032 (2016).
4. Brun, R.B. Experimental analysis of the eyeless mutant in the mexican axolotl (Ambystoma mexicanum). *Integrative and Comparative Biology*. **18** (2), 273–279, doi: 10.1093/icb/18.2.273 (1978).
5. Lopez, D. et al. Mapping hematopoiesis in a fully regenerative vertebrate: the axolotl. **124** (8), 1232–1242, doi: 10.1182/blood-2013-09-526970 (2019).
6. de Both, N.J. Transplantation of Axolotl Heads. *Science*. **162** (3852), 460–461, doi: 10.1126/science.162.3852.460 (1968).
7. Harrison, R.G. Some Unexpected Results of the Heteroplastic Transplantation of Limbs. *Proceedings of the National Academy of Sciences of the United States of America*. **10** (2), 69–74, doi: 10.1073/pnas.10.2.69 (2006).
8. Fields, E., French, V., Bryant, P.J., Bryant, S. V Pattern regulation in epimorphic fields. *Science*. **193** (4257), 969–981 (2013).
9. Gerber, T. et al. Single-cell analysis uncovers convergence of cell identities during axolotl limb regeneration. *Science*. **362** (6413), eaaq0681, doi: 10.1126/science.aaq0681 (2018).
10. Knapp, D. et al. Comparative transcriptional profiling of the axolotl limb identifies a tripartite regeneration-specific gene program. *PloS One*. **8** (5), e61352, doi: 10.1371/journal.pone.0061352 (2013).
11. Campbell, L.J. et al. Gene expression profile of the regeneration epithelium during axolotl limb regeneration. *Developmental Dynamics: an official publication of the American Association of Anatomists*. **240** (7), 1826–1840, doi: 10.1002/dvdy.22669 (2011).
12. Bryant, D.M. et al. A Tissue-Mapped Axolotl De Novo Transcriptome Enables Identification of Limb Regeneration Factors. *Cell Reports*. **18** (3), 762–776, doi: 10.1016/j.celrep.2016.12.063 (2017).
13. Gardiner, D.M. et al. Gene expression during the first 28 days of axolotl limb regeneration I: Experimental design and global analysis of gene expression. *Regeneration*. **2** (3), 120–136, doi: 10.1002/reg2.37 (2015).
14. Flowers, G.P., Timberlake, A.T., McLean, K.C., Monaghan, J.R., Crews, C.M. Highly efficient targeted mutagenesis in axolotl using Cas9 RNA-guided nuclease. *Development (Cambridge, England)*. **141** (10), 2165–2171, doi: 10.1242/dev.105072 (2014).
15. Smith, J.J. et al. A Chromosome-Scale Assembly of the Enormous (32 Gb) Axolotl Genome. *bioRxiv*. 373548, doi: 10.1101/373548 (2018).
16. Nowoshilow, S. et al. The axolotl genome and the evolution of key tissue formation regulators. *Nature*. **559** (7712), 50–55, doi: 10.1038/s41586-018-0141-z (2018).
17. Fankhauser, B.Y.G. The Effects of Changes in Chromosome Number on Amphibian Development. *The Quarterly Review of Biology*. **20** (1), 20–78, doi: 10.1086/394703 (1945).

614 18. Malacinski, G.M., Brothers, A.J. Mutant Genes in the Mexican Axolotl. *Science*. **184** (4142),
615 1142–1147, doi: 10.1126/science.184.4142.1142 (1974).
616 19. Armstrong, B. Gynogenesis in the mexican axolotl. *Genetics*. **83** (4), 783–792 (1976).
617 20. Flowers, G.P., Sanor, L.D., Crews, C.M. Lineage tracing of genome-edited alleles reveals high
618 fidelity axolotl limb regeneration. *eLife*. **6**, 1–15, doi: 10.7554/eLife.25726 (2017).
619 21. Shalem, O. et al. Genome - scale CRISPR - Cas9 knockout screening in human cells. *Science*.
620 **343** (6166), 84–87, doi: 10.1126/science.1247005.Genome-Scale (2014).
621 22. Wang, T., Wei, J.J., Sabatini, D.M., Lander E.S. Genetic Screens in Human Cells Using the
622 CRISPR-Cas9 System. *Science*. **343** (6166), 80–84, doi: 10.1126/science.1246981 (2014).
623 23. Yin, Z., Chen, L. Simple Meets Single: The Application of CRISPR/Cas9 in Haploid Embryonic
624 Stem Cells. *Stem Cells International*. **2017**, 1–6, doi: 10.1155/2017/2601746 (2017).
625 24. Khattak, S. et al. Optimized axolotl (Ambystoma mexicanum) husbandry, breeding,
626 metamorphosis, transgenesis and tamoxifen-mediated recombination. *Nature Protocols*. **9** (3),
627 529–540, doi: 10.1038/nprot.2014.040 (2014).
628 25. Vachon, P., Zullian, C., Dodelet-Devillers, A., Roy, S. Evaluation of the anesthetic effects of
629 MS222 in the adult Mexican axolotl (Ambystoma mexicanum). *Veterinary Medicine: Research*
630 *and Reports*. **7**, 1–7, doi: 10.2147/vmrr.s96761 (2016).
631 26. Montague, T.G. et al. Efficient Mutagenesis by Cas9 Protein-Mediated Oligonucleotide
632 Insertion and Large-Scale Assessment of Single-Guide RNAs. *PLoS One*. **9** (5), e98186, doi:
633 10.1371/journal.pone.0098186 (2014).
634 27. Moreno-Mateos, M.A. et al. CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9
635 targeting in vivo. *Nature Methods*. **12** (10), 982–988, doi: 10.1038/nmeth.3543 (2015).
636 28. Kumar, A., Simon, A. *Salamanders in Regeneration Research: Methods and Protocols*. Humana
637 Press. New York, NY (2015).
638 29. Hronowski, L., Gillespie, L.L., Armstrong, J.B. Development and Survival of Haploids of the
639 Mexican Axolotl, Ambystoma mexicanum. *Journal of Experimental Zoology*. **209**, 41–47 (1979).
640 30. Schreckenberg, G.M., Jacobson, A.G. Normal stages of development of the axolotl,
641 Ambystoma mexicanum. *Developmental Biology*. **42** (2), 391–399, doi: 10.1016/0012-
642 1606(75)90343-7 (1975).
643 31. Hertwig, G. Beitrage Zum Determinations- Und Regenerationsproblem Mittels Der
644 Transplantation Haploidkerniger Zellen. *Archiv f. Entwicklungsmechanik*. **111**, 292–316, doi:
645 https://doi.org/10.1007/BF02080951 (1927).
646 32. Fei, J.-F. et al. Efficient gene knockin in axolotl and its use to test the role of satellite cells in
647 limb regeneration. *Proceedings of the National Academy of Sciences of the United States of*
648 *America*. **114** (47), 12501–12506, doi: 10.1073/pnas.1706855114 (2017).
649



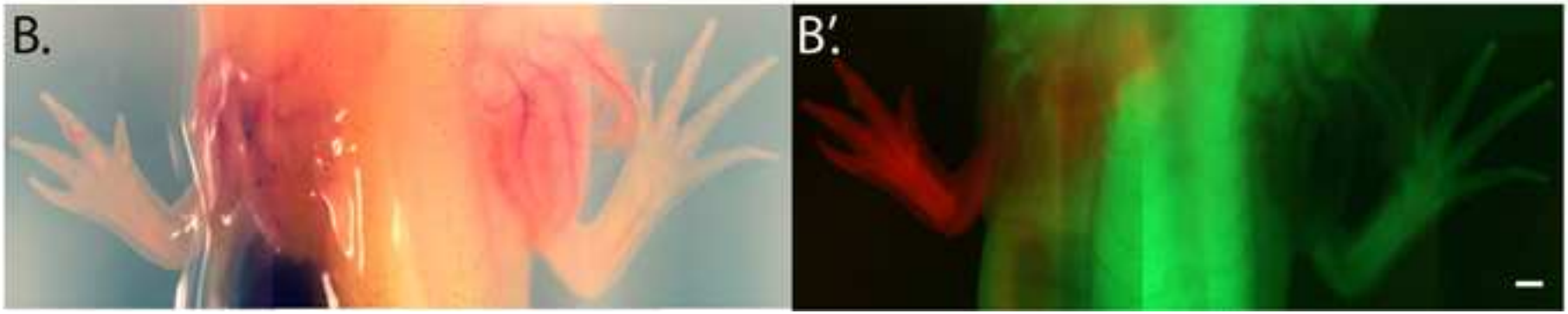
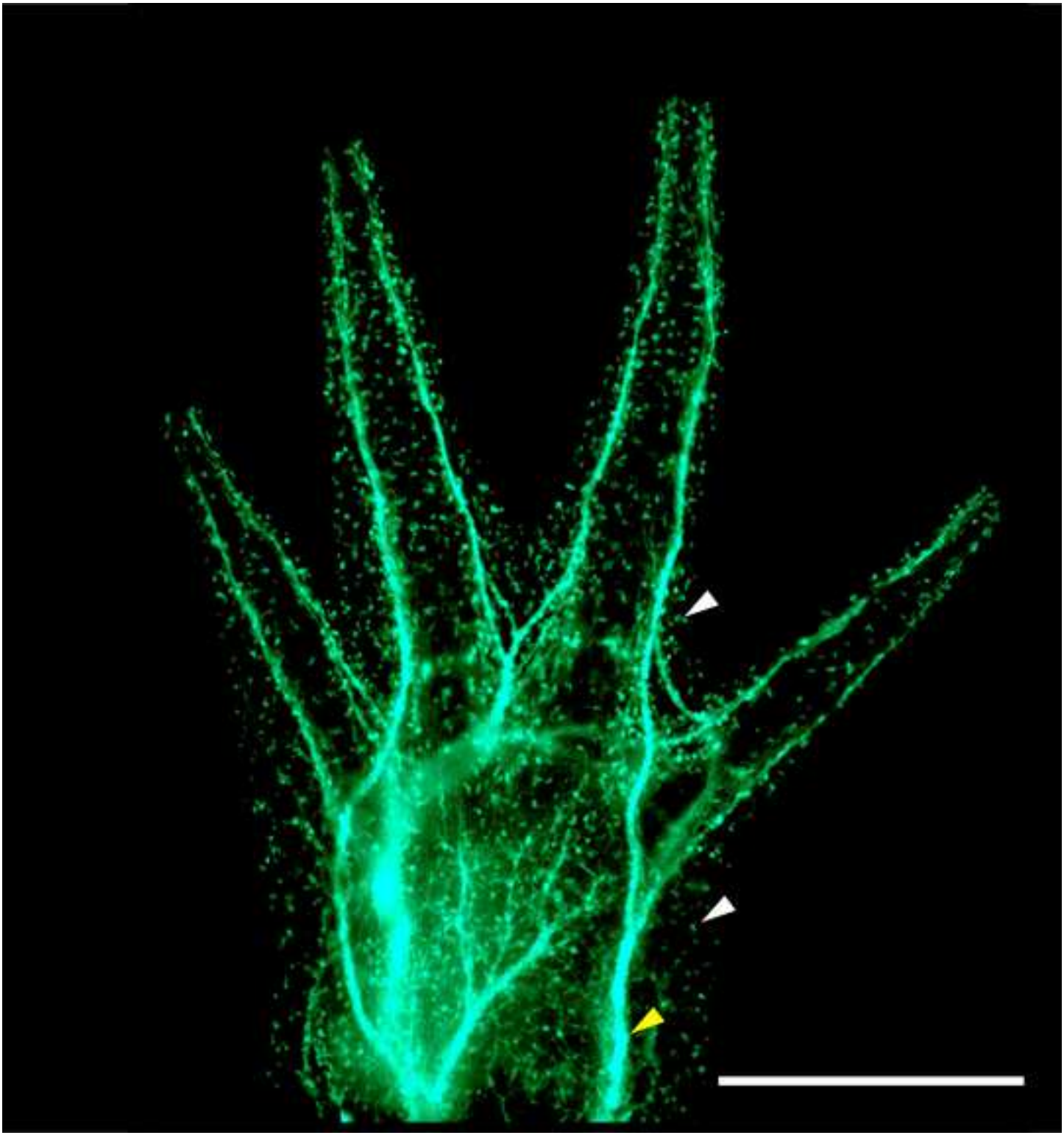
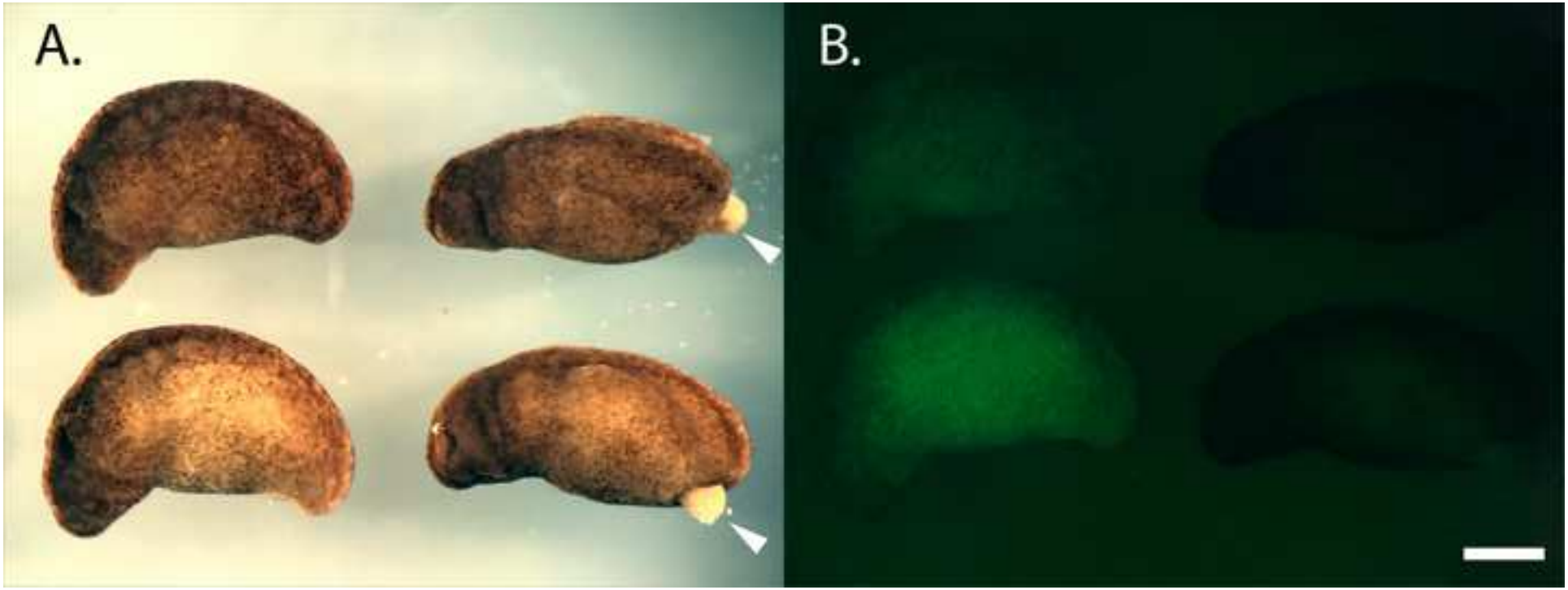
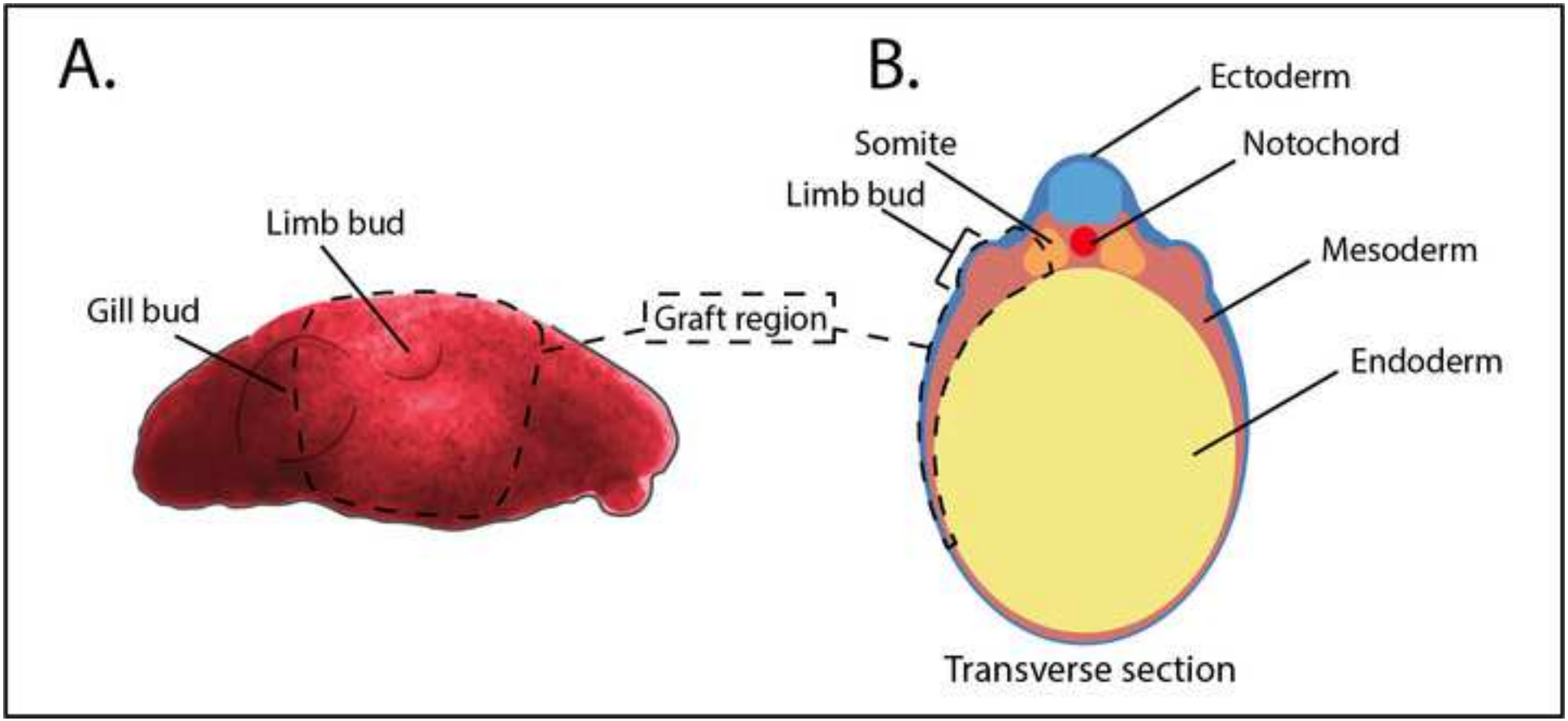


Figure 2 Revised

Click here to access/download;Figure;Figure 2 Haploid Limb Neural GFP.jpg







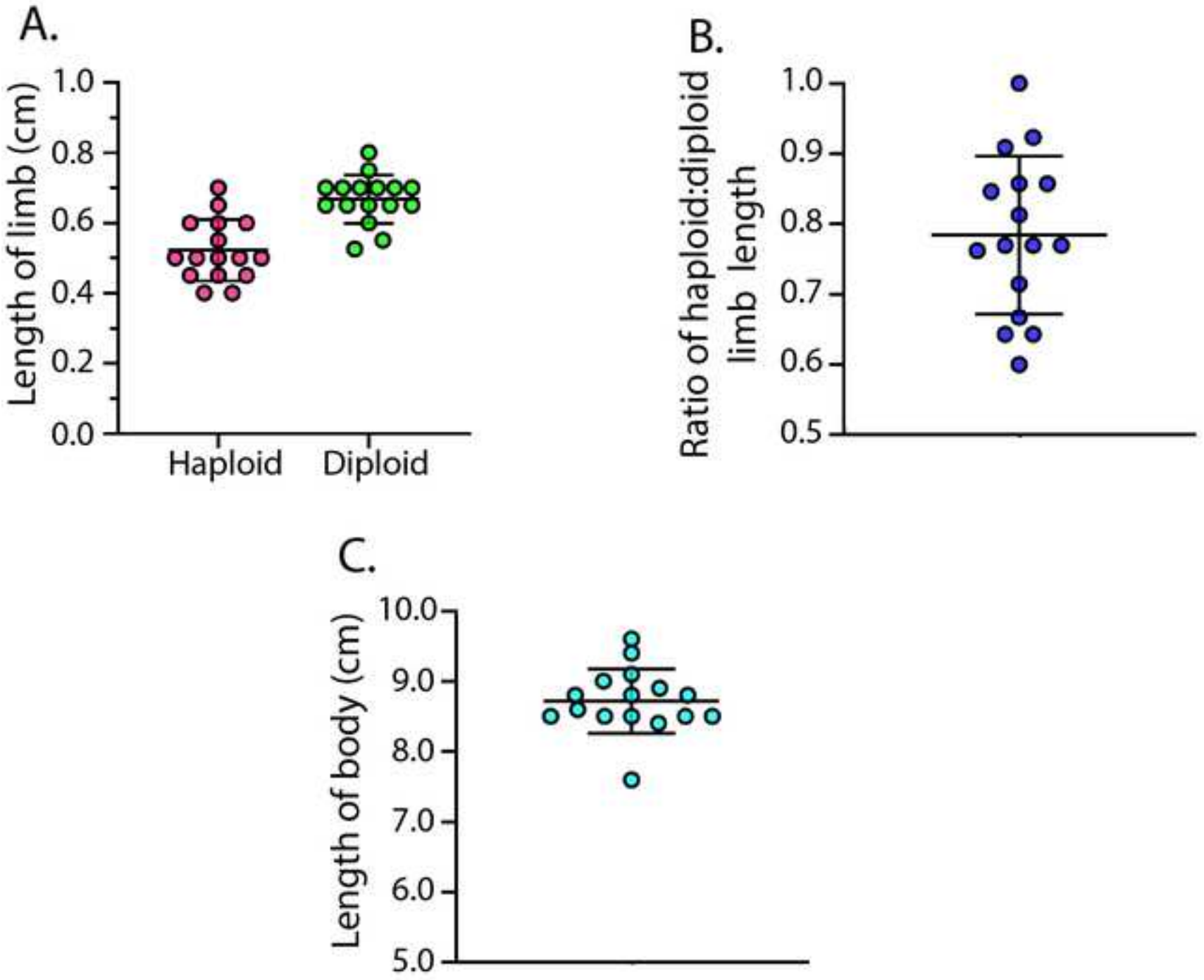


Figure 6 Revised

Click here to access/download;Figure;Figure 6 Regenerated Haploid and Diploid Limbs.jpg

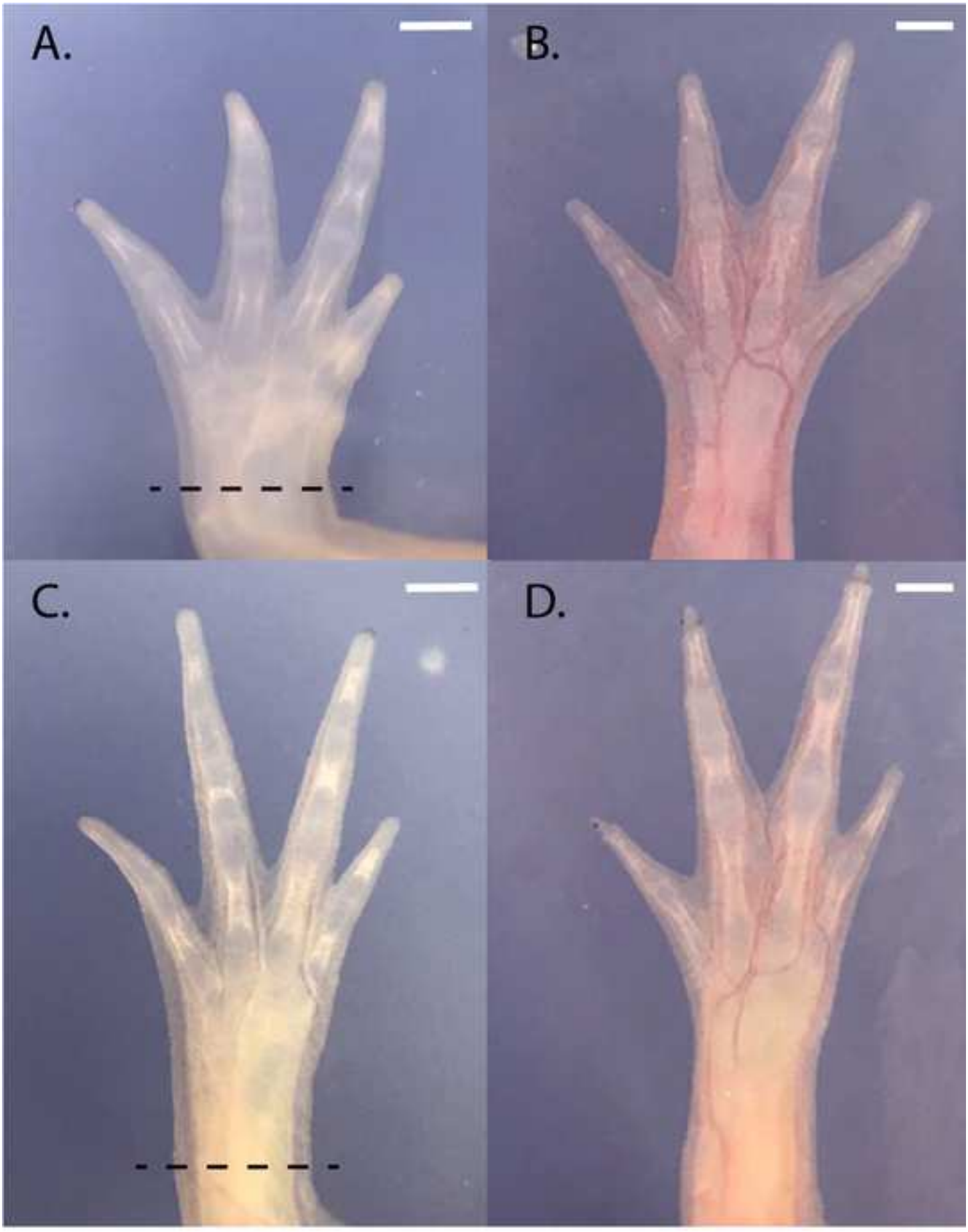


Figure 7 Revised

Click here to access/download;Figure;Figure 7 Normal vs abnormal haploid limbs CA.jpg

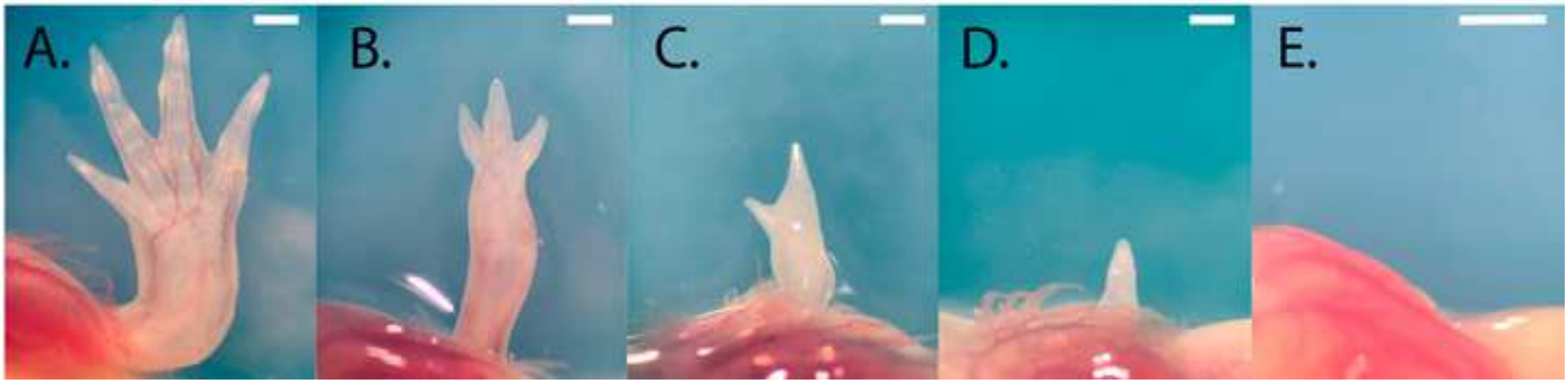
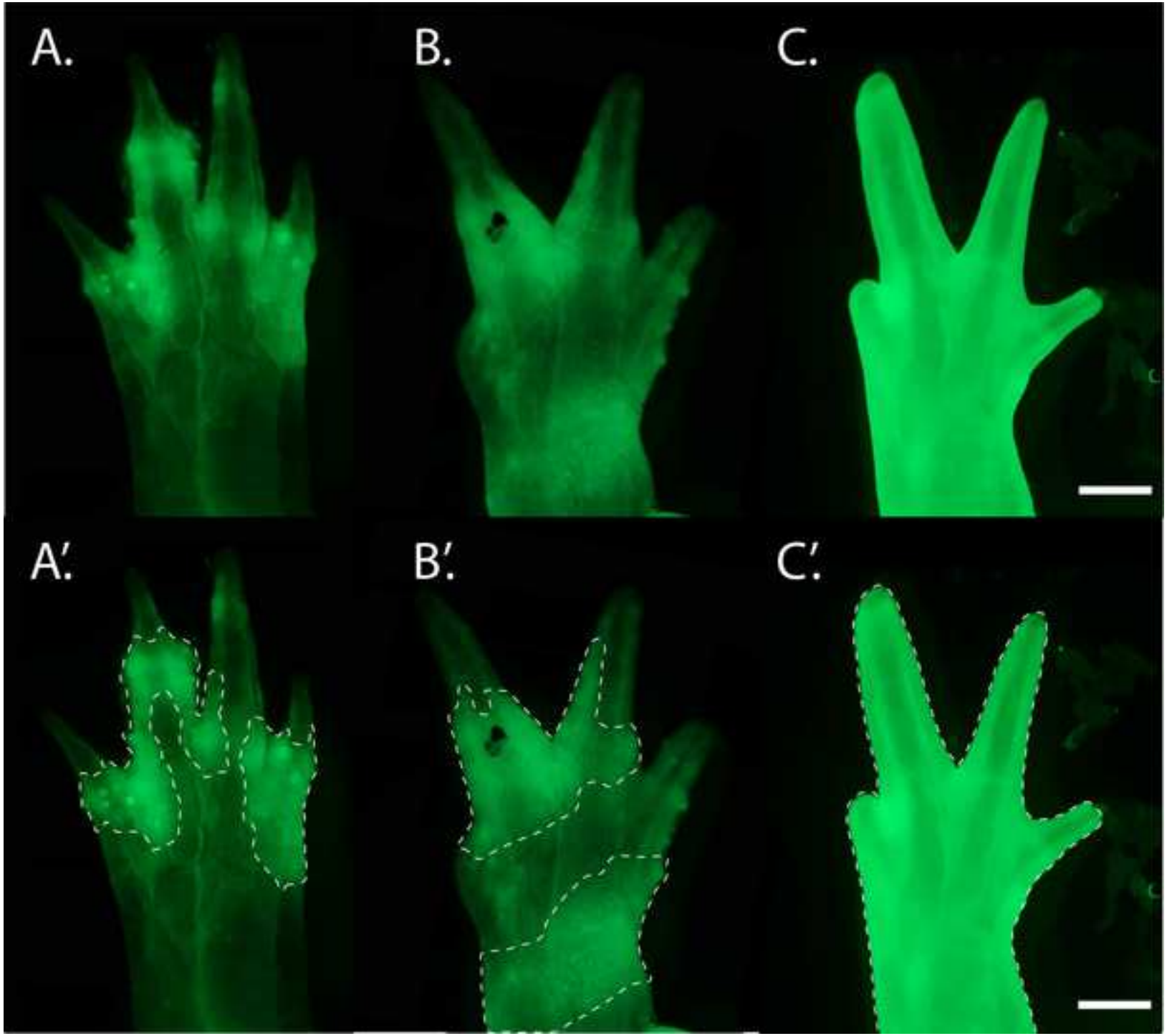
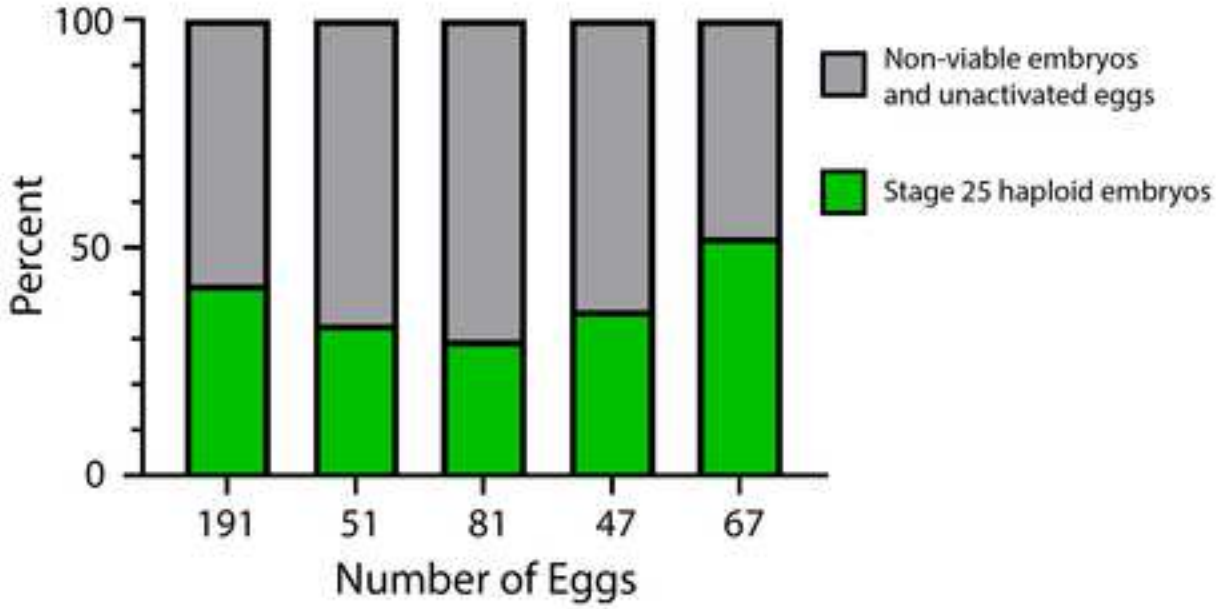


Figure 8 Revised

Click here to access/download;Figure;Figure 8 Variable Graft Purity.jpg



A. Percent Haploids Generated Using In Vitro Activation



B. Haploid Limb Graft Outcomes

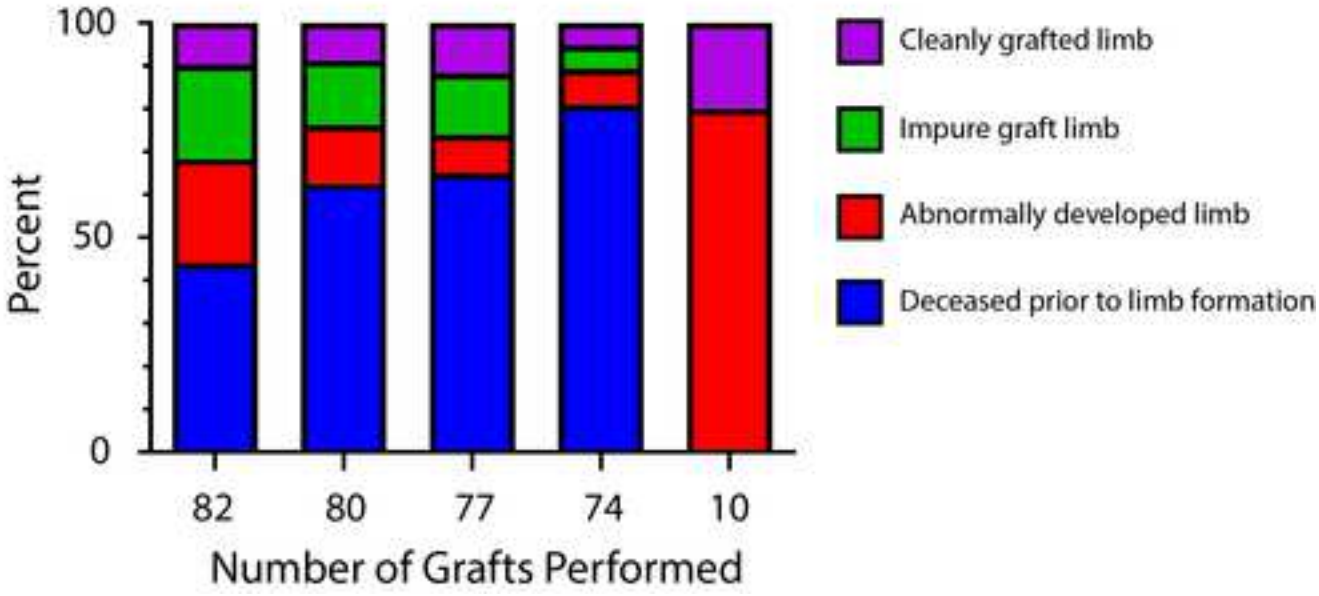


Table of Materials

Click here to access/download;Table of Materials;60156_Materials List 5_30_19.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
#55 Dumont Forceps	Fine Science Tools	11295-1	Only use Dumostar material (can be autoclaved)
Amphotericin B	Sigma aldrich	A2942-20ML	20 mL
Antibiotic-Antimycotic 100x	ThermoFisher	15240062	
Ciprofloxacin	Sigma aldrich	17850-5G-F	
Ficoll 400 (polysucrose 400)	bioworld	40600032-3	Ficoll 400
Gentamicin	Sigma aldrich	G1914-250MG	
Heating/Cooling Incubator	RevSci	RS-IF-233	
Human Chorionic Gonadotropin	Merk		Chorulon
Megascript T7 Transcription Kit	ThermoFisher	AM1334	40 reactions
Miroscope Cooling Stage	Brook Industries	Custom	Custom
NLS Cas9 Protein	PNAbio	CP01-200	4 vials of 50 µg protein each
Plasmocin	Invivogen	ant-mpt-1	Treatment level

Recipes

1.0x Marc's modified Ringer's solution (MMR)	0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM HEPES (pH 7.8), ph 7.4
40% Holtfreter's solution	20 mM NaCl, 0.2 mM KCl, 0.8 mM $NaHCO_3$, 0.2 mM $CaCl_2$, 4 mM $MgSO_4$, pH to 7.4

jove
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Generation of Chimeric Axolotls with Mutant Haploid Limbs through Embryonic Grafting

Author(s): Lucas D Sanor, G. Parker Flowers, Craig M Crews

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	CRAIG CREWS
Department:	MCDB
Institution:	Yale University
Title:	Professor
Signature:	[signature]
Date:	4/19/19

Please submit a **signed and dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Reviewers' comments:

Reviewer #1:

This is an interesting approach to generate chimeric axolotls for further studies. The protocols are well detailed and easy to follow.

In general the manuscript's protocols are very complete.

Thank you for your concise praise.

The intro would benefit from being a little more detailed in the advantages of producing such chimeric animals.

We have added several sentences in the introduction addressing these advantages.

There are references to a fig 7 which is not present in the manuscript that was provided for review.

We apologize for this omission and have added this figure (now labeled as figure 9).

There is also a reference to a video which was not available to the reviewer.

This was a reference to the yet-to-be-filmed video demonstration of embryonic grafting.

Overall this manuscript for JoVE is interesting and the grafting procedure especially could be very useful for many labs working with axolotls to study limb regeneration.

Reviewer #2:

The protocol will be useful for those examining tissue regeneration in axolotls and other similar systems.

Major Concerns:

1. Ln 233. Figure 7 is missing and cannot be located in the text.

2. Ln 264, Figure 6. It is unclear what each arrow points. Perhaps use dotted lines to highlight the areas. The legend should describe what each panel A, B and C is.

We have included this figure, and made the suggested improvements to Figure 6 (now Figure 8) and its caption.

Minor Concerns:

1. Abstract: "We combine several embryological and genetic techniques, including haploid generation via in vitro activation, CRISPR/Cas9 mutagenesis, and tissue grafting into one protocol to produce a unique system that is, to our knowledge, the first platform for haploid genetic screening in a vertebrate." It is not necessary to state whether this approach is unique and the first platform.

This statement has been removed.

2. Ln 34-36. "…other essential processes" is vague. Please cite some examples.

We have mentioned specific processes in the abstract and further explanation in the discussion.

3. Ln 73-75. Indicate the pH of HEPES buffer.
4. Ln 295-297: Describe what Holfreter's solution is.
5. Ln 178-179. Final concentrations of Plasmocin, Amphotericin B and Ciprofloxain are needed.

We have made these changes.

Reviewer #3:
I think this will be a great resource for others in the field even if they only have interest in singular portions of the protocol. I think it is very thorough and my only suggestions are related to giving more context and information to the reader to understand why certain techniques are being used (haploid embryo creation) and more complete information on timing and input/output (e.g. how many embryos go into a step and what is the relative viability afterwards). I think this would make this manuscript an even more fantastic resource for the community.

We have significantly improved the manuscript by incorporating your suggested changes. We agree that independent components of this protocol will have additional value to axolotl researchers.

Major critiques
- The authors should expand with more context and background about the historical creation of haploid animals

We have included additional description of the historical precedent and use of haploid salamanders in the introduction.

why haploid animals are advantageous or needed. This could be based on the authors clear expertise in performing CRISPR analysis. I imagine they could give an example of how often they observe mono- versus bi-allelic mutations which might necessitate the need to have haploid animals.

Further discussion of the advantages of working with haploid animals is included in both the introduction and discussion.

-A flowchart or detailed timeline would be helpful as there are several concurrent things happening and it would help the user prepare the timing of different steps. This seems critical to make sure you have appropriately match donor and host embryos.

We have added a flowchart as Figure 1A and additional details within the protocol and discussion for guidance in stage-matching donor and host embryos.

There needs to be some viability measures for each part of the protocol. How many embryos do you typically get surviving after haploid activation, CRISPR injection, grafting, etc. I think it's important for the user to get a good impression of what they will actually be able to achieve starting from a single clutch of 100-200 oocytes. Although I am sure this will vary depending on the skill of the experimenter and the luck of oocyte collection, it would be nice if the authors can provide in tabular form what they produced for several replicates of their protocol at each step.

We have included multiple replicates in graphical form of oocyte activation experiments. We have also included several replicates of outcomes of grafting experiments with successfully activated haploids (we failed to properly upload in the previous submission). While we do not have precise survival curves for these experiments, we have noted that the loss of grafted animals occurs at both late embryonic and larval stages (in the depicted examples, death was almost entirely in feeding larvae). In our own experience with rearing larval axolotls, there is significant and difficult-to-control variation in the viability of animals.

How do the authors distinguish haploid phenotypes versus CRISPR-edited phenotypes? This should be discussed.

We have included notes, figures, and discussion regarding the features of the characteristic haploid limb phenotype. We include further discussion regarding the need to preselect cleanly grafted haploid limbs for phenotypic analysis.

Do the sizes of the limb and speed of regeneration remain consistent between limbs regardless of ploidy?

We included data and figures showing the quality of haploid limb regeneration. As we have discussed with the editor, we have a current manuscript in review describing lineage tracing analysis of haploid limbs and more extensive characterization of the rate and fidelity of haploid limb regeneration.

As well, the authors should discuss the limitations that still exist with their method. I would imagine that there are still genes that remain inaccessible to CRISPR editing due to their crucial role in early embryogenesis such as BMPs, Wnts, etc. In these cases an inducible system would be needed.

We have included additional discussion describing the value of inducible genetic systems, the merits and limitations of this haploid approach, and potential methods to investigate the role of essential genes in regeneration in haploid axolotl limbs outside of that outlined in this protocol.

- Line 164 - Is there a reason that the authors de-jelly everything before visually sorting the embryos for GFP positive and negative? That sounds like a lot of extra work.

While we have both dejellied before and after sorting for GFP+ embryos, we have adjusted the protocol to recommend dejellying only GFP+ embryos.

- Figure 1 - A brightfield image is really needed. I can't really see or compare haploid to diploid limbs.

We have replaced the original image of a grafted animal and included a corresponding bright-field image.

- Figure 3-6 should indicate the embryonic stage or animal stage.

We have indicated these stages.

- The authors indicate in Figure 5 that abnormal limb development can occur after the procedure. Are these due to problems with ploidy or with the grafting? Do the authors ever perform diploid-diploid grafts as controls? This should be mentioned.

We have added discussion to indicate that limb developmental abnormalities are the result of incomplete grafts, as limbs that do not contain extensive host contributions do not display abnormalities. While we have performed numerous diploid-diploid grafts while developing this technique and observed that impure grafts were associated with limb developmental abnormalities, we do not have data from parallel experiments to compare the outcomes of these grafts to haploid-diploid grafts.

- I did not receive a Figure 7, although it was described in the figure legend.

This figure, which was erroneously omitted, is now Figure 9.

Reviewer #4:

This work therefore represents a distinctive approach from the currently available tools for gene function analysis in the axolotl and may provide a time and cost-optimized alternative for gene screening analysis in the limb system, hence meriting publication. The step-by-step protocol provided is detailed, clear and concise. Furthermore, the steps proposed for video record cover the most critical aspects of the methodology.

-In line 227, the authors mention that "haploid limbs are smaller than their diploid counterparts". This distinctive phenotype is not further described nor adequately represented. Are the haploid limbs consistently generated with the same relative size between animals? It would help to visualize this by improving Figure 1 to either outline the contralateral limb or to provide a bright field picture clearly depicting both limbs. Also, quantifications of this phenomenon should be provided.

We have included notes, figures, and discussion regarding the features of the characteristic haploid limb phenotype. We include further discussion regarding the need to preselect cleanly grafted haploid limbs for phenotypic analysis.

-The use of GFP+ hosts allowed the authors to identify an inherent limitation of this technique, which is the host-derived innervation and vasculature present in the haploid limb. Importantly, it also provides a mean to evaluate the extent of tissue contribution from the diploid host tissues to the haploid limb, which is variable among samples. The text mentions that this information and the proportion of normally developed haploid limbs achieved is represented in Figure 7, which is unfortunately missing and must be included for publication.

This figure, which was erroneously omitted, is now Figure 9.

The only reference to regenerates is found in the discussion, line 331, which simply states that they regenerate normally. This is insufficient to show that indeed this technique can be used for studying limb regeneration. Could the authors please elaborate or provide more information regarding e.g. timing, size and morphology and frequency of eventual alterations found in regenerates derived from the chimeric limbs, in regard to their diploid counterparts and/or to the

original haploid limb? Importantly, observed variances should be reported/commented. The verification of a consistent pattern of regeneration within haploids and between haploid and diploid samples would greatly aid supporting the validation of this approach for the intended purpose of studying regeneration.

We have included additional data describing the development and regeneration of haploid limbs, and a figure depicting a fully regenerated haploid limb. As we have discussed with the editor, we have a current manuscript in review describing lineage tracing analysis of haploid limbs and more extensive characterization of the rate and fidelity of haploid limb regeneration.

-In general, legends should be improved to convey the depicted information more clearly.

We have modified all figure legends.

Figure 6 - It is not clear to which tissues the white arrows refer to, and therefore the picture should be improved in this regard.

We have provided the recommended outlines (now Figure 8)

-Table of material/reagents - recipes for solutions (e.g. surgical media) may be included along with the materials list.

These recipes have been included.

-Figure 5 - Would it be possible to outline the haploid outgrowth on E, for a clearer visualization?

We have modified the caption to indicate that there is no limb outgrowth (this is now figure 7).

-A couple of general comments referring to protocol details are mentioned in the discussion, specifically in lines 278-281, which may be considered to include in the protocol section instead. Alternatively, more extensive comments in the protocol might be added as notes to the protocol instead.

We have added extensive notes to the protocol.

-in the first part of the protocol, lines 80-83, the authors mention they place an adult female at 8-10°C for 48h for haploid embryo generation. This seems rather harsh - is this strictly necessary or is there any other alternative?

This step prevents rapid egg laying after induction of ovulation, but is not absolutely required for experimental success. We have performed this step at higher temperatures (e.g., 12°C) and have altered the protocol to reflect this.