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Protocol for lens removal in embryonic fish and its application on the developmental effects of eye regression

Luis Espinasa[1], Marie Pavie[2], Sylvie Rétaux[2]

1 *School of Science, Marist College, 3399 North Rd, Poughkeepsie, New York 12601, USA* **2** *Paris-Saclay Institute of Neuroscience, CNRS and University Paris-Saclay, Saclay, France*

Corresponding author: Luis Espinasa (luis.espinasa@marist.edu)

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Abstract

The lens plays a central role in the development of the optic cup. In fish, regression of the eye early in development affects the development of the craniofacial skeleton, the size of the olfactory pits, the optic nerve, and the tectum. Lens removal further affects olfaction, prey capture, and aggression. The similarity of the fish eye to other vertebrates is the basis for its use as an excellent animal model of human defects. Questions regarding the effects of eye regression are specifically well-suited to be addressed by using fish from the genus *Astyanax*. The species has two morphs; an eyeless cave morph and an eyed, surface morph. In the cavefish, a lens initially develops in embryos, but then degenerates by apoptosis. The cavefish retina is subsequently disorganized, degenerates, and retinal growth is arrested. The same effect is observed in surface fish when the lens is removed or exchanged for a cavefish lens. While studies can greatly benefit from a control group of surface fish with regressed eyes brought through lensectomies, few studies include them because of technical difficulties and the low survivorship of embryos that undergo this procedure. Here we describe a technique with significant modification for improvement for conducting lensectomy in one-day-old *Astyanax* and other fish, including zebrafish. Yields of up to 30 live embryos were obtained using this technique from a single spawn, thus enabling studies that require large sample sizes.

Keywords

Eye regression, Lemsectomy, Sierra de El Abra, Stygobite, Troglobite, Troglomorphy

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Introduction

The lens plays an important role in the development of the optic cup (Thut et al. 2001; Yamamoto and Jeffery 2001). Using fish from the genus *Astyanax*, questions regarding the effects of eye regression can be addressed. The species has two morphs; an eyeless cave morph and an eyed, surface morph that remain inter-fertile. The blind Mexican tetra is a model system in evolutionary developmental biology which has provided an unprecedented understanding of the genetic and developmental controls of features associated with being eyeless and living in the continuous darkness of the caves (Jeffery 2001). *Astyanax* are well fitted for laboratory research and suitable for experimental manipulation due to several advantageous characteristics, including small size, high fecundity, short lifecycle, and relative ease of care. Since the genome of both surface and cave morphs has been sequenced, abundant genetic and molecular resources are available to support research in *Astyanax* (McGaugh et al. 2014; Imarazene et al. 2021; Warren et al. 2021). In addition, the similarity of the fish eye to those of other vertebrates provides the basis for its use as an excellent animal model of human eye defects (Schmitt et al. 1994; Malicki 2000; Malicki et al. 2002; Avanesov and Malicki 2004; Cavodeassi and Wilson 2019).

The role of the lens in eye development has been studied in *Astyanax*. Lens development occurs rapidly in this species. By 18.5 hours post-fertilization (hpf), the *Astyanax* lens has rounded from the placode and is visible (Hinaux et al. 2011; Devos et al. 2021). A lens and a layered retina initially develop in cavefish embryos, but the lens rapidly undergoes massive apoptosis after one day of development (Yamamoto and Jeffery 2000). The cavefish retina is subsequently disorganized, apoptotic cells appear, the photoreceptor layer degenerates, and retinal growth is arrested (Alunni et al. 2007; Strickler et al. 2007). When a surface fish lens is transplanted into a cavefish's developing optic cup, it stimulates growth and development, restoring optic tissues lost during cavefish evolution. Conversely, eye growth and development are retarded following transplantation of a cavefish lens into a surface fish optic cup or through a lensectomy early in development (Yamamoto and Jeffery 2000). These results show that evolutionary changes in signal(s) from the lens are involved in cavefish eye degeneration.

Manipulations of eye formation by transplantation of the embryonic lens or by lensectomy have been crucial to understanding eye-dependent and eye-independent processes. Cavefish craniofacial skeletons and the size of the olfactory pits in adults were found to correlate with eye development (Yamamoto et al. 2003). Likewise, the lens indirectly influences the optic nerve and tectum development in blind cavefish (Soares et al. 2004). Lensectomy studies can also help inform the effect of developing eyes on behavior. For example, surface fish raised in the dark or after embryonic lens ablation leading to eye degeneration have improved olfactory detection capacities (Blin et al. 2018). However, when analyzing prey capture competition assays on these fish, the eye-dependent developmental processes were found not to be the main determinant for enhanced prey capture skills (Espinasa et al. 2014). Aggression is another example. Surface-dwelling individuals are highly aggressive, whereas their blind, cave-dwelling counterparts tend to show little or no aggressive behavior. Surface fish blinded early in their embryonic development through lensectomy remain highly aggressive as

adults (Espinasa et al. 2015). Thus, aggression in *Astyanax* can be triggered without visual stimuli in surface fish.

In this paper we describe the technical approach for removing a lens from a developing *Astyanax* surface morph that is generalizable to other species. Although this technique has been used in the past, prior studies reported low survivorship and a substantial degree of difficulty. The modified protocol described herein allows for much faster and more reliable removal of lenses that can yield high survivorship. We aim to provide a clear roadmap for other interested researchers to perform this experimental technique.

Protocol

Animals were treated according to the French and European regulations for the use of animals in research. SR's authorization for using animals in research, including *Astyanax mexicanus*, is 91–116. The Paris-Saclay Institute's animal facility authorization number is B91-272-108. Specimens are those used in Espinasa et al. (2014) and Blin et al. (2018).

Part 1: Preparing the embryos

Astyanax breeding has been described elsewhere (Elipot et al. 2014; Peuß et al. 2019; Ma et al. 2021). Briefly, an increase of temperature from 22 °C to 26.5 °C in the first three days of a breeding week provides two-to-three consecutive spawning days with maximal numbers of high-quality embryos, which is then followed by a decrease of temperature from 26.5 °C to 22 °C during the last three days of the spawning week. Most spawning occurs at night for both surface and cavefish. In the morning, collect embryos in 100 × 15 mm Petri dishes, sort and clean them, and transfer them into embryo medium (EM-Westerfield 2000) with methylene blue. An alternative is to conduct in-vitro fertilization.

Keep the embryos in EM in a 23 °C incubator until the desired stage. The lens becomes visible in *Astyanax* kept at 23 °C at 18.5 hpf. Hatching occurs at 24.5–28 hpf. The lens enters apoptosis at about 25 hpf. When conducting lensectomy on surface fish to replicate the effects of lens degeneration in cavefish, the optimum time is 1–3 dpf, or within 48 hrs after hatching. If lensectomy is to be conducted before hatching, remove the chorion manually with two pairs of sharp forceps, and incubate the embryos in 0.2% EDTA in Calcium-free Zebrafish Ringer's (ZFR) for 30 minutes.

Part 2: Preparing dissection needles

In previous protocols, two needles were used. One with a blunt tip needle made of a thin tungsten wire and a second one with a sharp tip made by holding the tungsten wire over a Bunsen burner for 1–1.5 minutes, burning off the metal, and creating a very fine tip). Previous protocols instructed lensectomies to be conducted by hand under a microscope. Since the lens is only about 50 μm, extreme precision is required to

ablate the lens without harming other structures. Normal tremor of the hands makes this extremely challenging, even for highly trained people.

In this improved protocol, instead of using tungsten needles held by hand, microinjection needles were made from glass capillaries mainly with a Narishige's PC-10 Dual-Stage Glass Micropipette Puller, with the puller was set to a one step weighted pull at 70.5 °C. Other brand micropipette pullers were tested and found to give similar results. Borosilicate glass capillaries are heated and pulled to get extremely fine and sharp needles, similar to those used for cell injections (Fig. 1A, B). These needles are then attached to a manual micromanipulator (Type MM33 Rechts; Märzhäuser, Wetzlar, Germany). The micromanipulator allows for precise movements in the X, Y, and Z directions, eliminating hand tremor effects and maximizing the precision of movements. We found that the best technique was with one hand controlling the micromanipulator, while the other hand moved the petri dish that holds the specimen (Fig. 1C). This combination allowed for the best combination of dexterity and efficiency of movements.

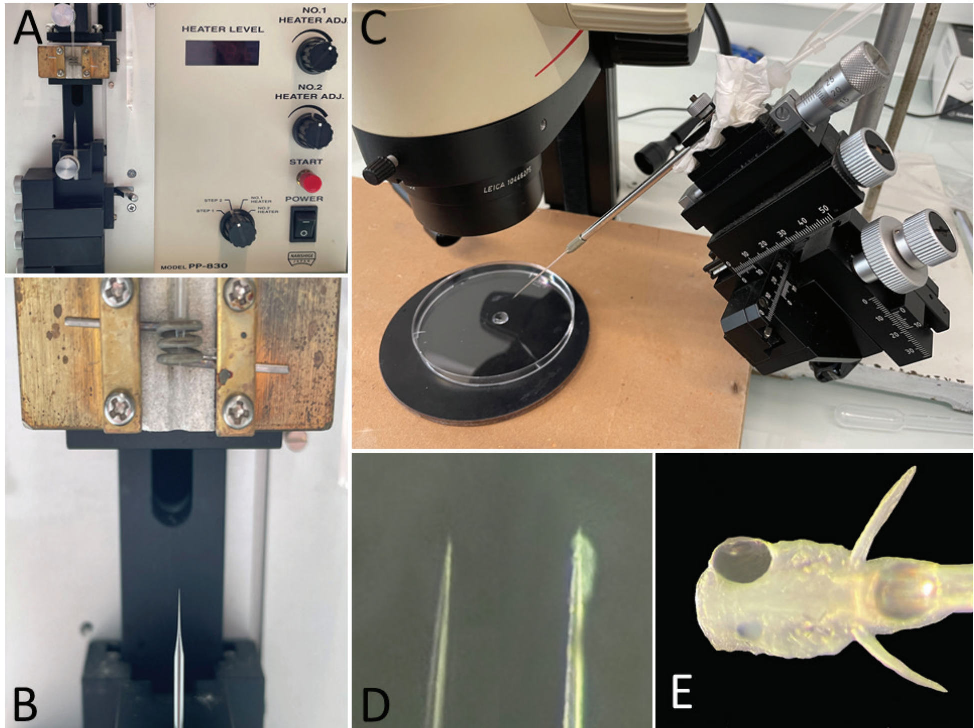

Figure 1. A for the preparation of dissection needles, microinjection needles are made from borosilicate glass capillaries with a Micropipette Puller **B** glass capillaries are heated and pulled to get extremely fine and sharp needles **C** instead of manipulating the dissection needle by hand, a micromanipulator is used. This dramatically reduces jittery movements that can puncture neighboring structures such as the brain or the heart. The micromanipulator allows precise puncturing around the lens with the needle's movements controlled easily at less than 5 µm **D** clean needles are essential. Throughout the procedure, the needle progressively gets covered with a fatty substance that essentially blunts the needle. It is best to exchange it for a new one **E** embryo a week after a one-sided lensectomy in dorsal view. The left, lensectomized eye is regressing.

Part 3: Preparing reagents and equipment

- Embryo medium (EM; pH 7.0, per liter, contains: 10 ml Hanks Solution #1, 1 ml Hanks Solution #2, 10ml Hanks Solution #4, 10 ml Hanks Solution #5, 0.35 g sodium bicarbonate, 300 uL of 2M HCl, penicillin-streptomycin 500,000U) As in The Zebrafish Book (University of Oregon Press, 2000).
- Anesthetizing solution:10 ml EM, 1 mg ms-222 (tricaine methanesulfonate), 1 mg $NaHCO_{3.}$
- 2% low melting agarose: 10 ml EM, 1 mg ms-222 (tricaine methanesulfonate), 1 mg $NaHCO_3$, 0.2 gr low melting agarose.
- Pulled glass capillaries.
- Dissection needle with a thin tungsten wire blunt tip.
- Mini scalpel.
- Plastic pipette dropper (wide mouth so the embryo can easily fit).
- 200 µl pipette.
- Petri dishes.
- Dissection microscope.
- Manual micromanipulator.

Part 4: Lensectomy procedure

• At the desired stage (40 hpf, for example), take the embryos kept in EM in the incubator. With a plastic pipette dropper, carefully transfer the embryos to the anesthetizing solution and incubate for about 30 seconds or until the embryos stop moving. The number of embryos depends on proficiency. We operated up to ten specimens simultaneously, but for best survival and efficiency, about three at a time is recommended.

• With the pipette dropper, transfer anesthetized specimens to a petri dish (Fig. 2A). Before adding the agarose, absorb the excess liquid (Fig. 2B). It was found that, otherwise, the agarose surrounding the embryo would be diluted and would not grasp as firmly during the micromanipulations. However, caution should be used that the embryo does not dry before adding the agarose.

• Add the 2% low melting agarose when the melted agarose is close to room temperature but before it solidifies on and around the embryo (Fig. 2C). Most embryos will naturally lie on their sides in the agarose. Reorient those that do not lie in this position so that one eye is facing up before the agarose solidifies. The depth at which the embryo lies within the agarose drastically changes the performance. When very deep, it is difficult to see the structures, and when traversing the agar, the needle gets deflected from its target. When barely covered, it risks detaching the specimen from the agar. We found that adding about 100 µl agarose worked well, but sometimes excess agarose must be reabsorbed when the specimen sinks or is attached to the petri dish.

• Wait for the 2% agarose to solidify. For time efficiency, the Petri dish containing the samples can be put in a refrigerator for 1–2 min. It was also found convenient to use this time to make a pair of microinjection needles. After the agar solidifies, the Petri dish is transferred under a dissecting microscope (Fig. 1C). Microscopes of high quality,

good resolution, and good illumination are recommended for the best results. By its side, there will be the manual micromanipulator with the microinjection needle attached to it (Fig. 1C). Adding a few drops of liquid anesthetizing solution on top of the agarose is optional. Depending on the specific sample, it can enhance or reduce visual clarity.

- Using the microinjection needle, very carefully cut the lens out from the embryos using small strokes. Make sure to cut just close enough to the lens that it neither tears it, nor removes too much tissue from the rest of the eye. We developed three styles, each with its advantages and disadvantages:

Style 1: With the micromanipulator, slowly bring down the needle by the side of the lens. Pressure down until it makes a puncture in the surface ectoderm/cornea at the junction between the lens and optic cup (Fig. 3A). Depending on the specimen and how solid the

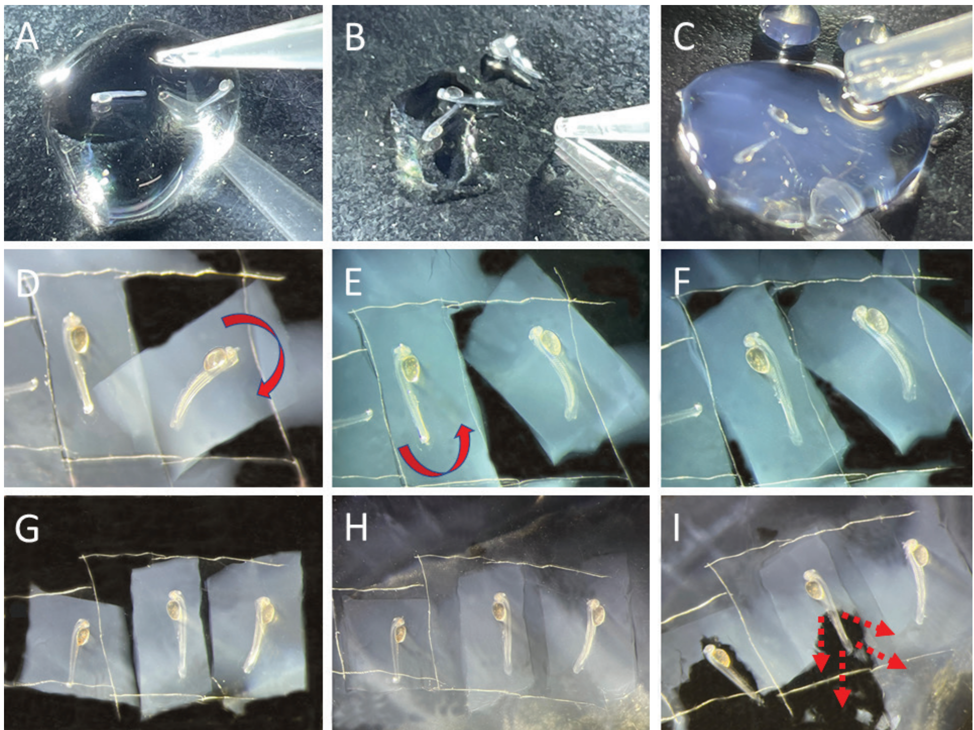

Figure 2. A specimens are transferred to a Petri dish after being in anesthetizing solution for 30 seconds or until embryos stop moving **B** absorb the excess liquid **C** add 2% agarose EM. The depth at which the embryo lies should not be too deep because the needle gets deflected from the target, and it is difficult to see the structures. However, it risks detaching the specimen from the agar when barely covered. After agarose solidifies, proceed with lensectomy **D–G** if lensectomy is to be done on both sides, with a scalpel, cut a rectangle of the agar around the specimen. Very gently slide the scalpel under the rectangle of agar with the specimen. Helped with twicers, flip around the agar slab, so the specimen is on the other side. More than one specimen can be done at a time to increase yield **H** add 2% agarose around the rectangle of agar or over the specimen if it was dislodged **I** after the second lensectomy is done, submerge in embryo media and gently dislodge the embryo from the agar with downward strokes starting around the tail and ending on the head.

agarose grabs the specimen, the eye can deform and sink slightly down with the pressure until the needle ruptures the tissue. Be careful that the needle does not go further down and punctures other structures, such as the brain or the heart. The use of the micromanipulator is a great improvement to the previous protocols that used a hand-held needle in this regard. After a hole is made with the micromanipulator, pull the needle out. With the hand holding the petri dish, slightly reposition the sample so the needle can make a different puncture at another site near the lens (Fig. 3B). Repeat these punctures with a circling pattern around the lens. Ten to fifteen punctures are performed (Fig. 3C). Afterward, with a coordinated motion of the hand holding the petri dish and the micromanipulator, insert the needle between punctures and gently pull the needle out to tear the tissue between punctures. Repeat this progressive tearing of tissue around the lens. Finally, put the needle on one side, under the lens, and push it out of the optic cup

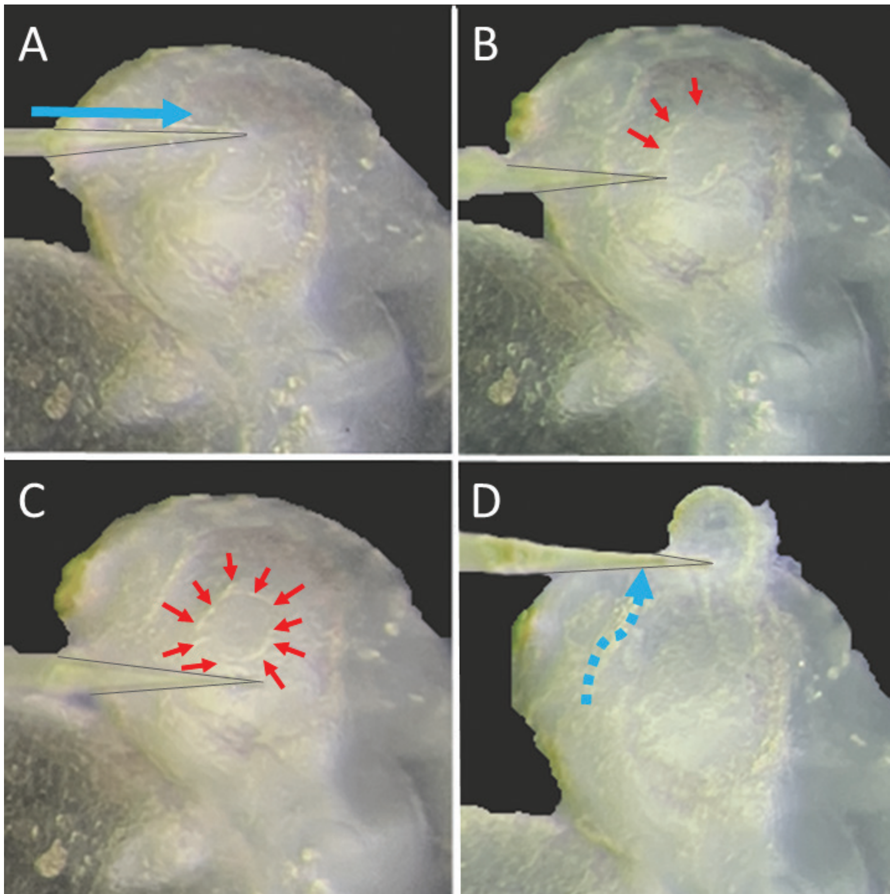

Figure 3. Style #1 for doing lens ablations **A** with the micromanipulator, slowly bring down the needle by the side of the lens and pressure down until it makes a perforation on the surface ectoderm/cornea **B** repeat these punctures around the lens **C** insert the needle between punctures, and with a coordinated motion of the hand holding the petri dish and the micromanipulator, gently pull the needle out to tear the tissue between punctures **D** put the needle on one side, under the lens and push it out of the optic cup.

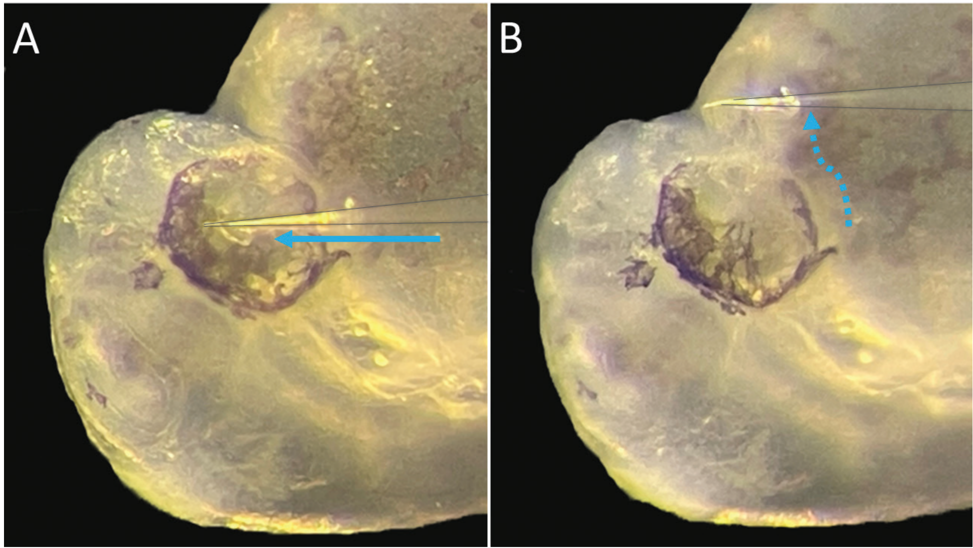

Figure 4. Style #2 for doing lens ablations **A** with the micromanipulator, slowly bring down the needle by the side of the lens and puncture the surface ectoderm. While inside the optic cup, position the needle so that it is under the lens **B** with a coordinated motion of the hand holding the Petri dish and the micromanipulator, move the needle away from the eye with the lens position in the center. The optic cup will distend until it rips open, with the lens bursting out of the eye cup.

(Fig. 3D). It is common for the lens to brake during this step, especially if no liquid has been added above the agar. With the needle, any lens remains can then be scooped out.

Style 2: With the micromanipulator, slowly bring down the needle by the side of the lens and puncture the surface ectoderm. While inserted, position the needle so that it is under the lens (Fig. 4A). Afterward, move the needle away from the eye with the lens positioned in the center. This is done with a coordinated motion of the hand holding the Petri dish and the micromanipulator. The optic cup will distend until it rips open, with the lens bursting out of the eye cup (Fig. 4B). In most cases, the lens will be obliterated. With the needle, any lens remains can then be scooped out. Style #2 is riskier because, depending on the robustness of the specimen and the grip the agarose has on the specimen, if the ripping of the tissue is done too fast, it can dislodge other internal tissues and kill the specimen. If the agar cover is not too deep, the specimen can also detach from the agar before the lens is ripped out. We found that experience and gentle slowness while pulling was necessary to perform this style effectively. Once this style is mastered, time dedicated per lensectomy is shortened without significantly reducing survival.

Style 3: With the micromanipulator, slowly bring down the needle just by the side of the lens, closer than the previous two styles, and puncture the overlying ectoderm. Position the needle so that it is above the lens instead of below (Fig. 5A). Gently scrape the overlaying agarose and scrape the tissue over the lens. The tissue will tear, and the lens will float up if there is liquid (Fig. 5B). It may need some nudging with the needle.

- If lensectomy is to be done on only one side of the face, submerge the agar containing the specimen in embryo medium and gently dislodge the embryo from the agar; with a blunt tip dissection needle made with a thin tungsten wire or the hair of a toothbrush, stroke downward the agar around the embryo starting around the tail. Pieces of agar will dislodge. Progressively dislodge fragments of agar from tail to head until the specimen is released (Fig. 2I). Caution should be used to minimize touching the specimen, especially with the tip of the needle. Alternatively, fine-tip twicers can dislodge agar on either side of the embryo, again, from tail to head. Once free, the embryo should be transferred to clean embryo medium and put in the incubator.
- If lensectomy is done on both sides, it is best to have the agar dry, not overlaid with liquid. With a scalpel, cut a rectangle of the agar around the specimen (Fig. 2D). Very gently slide the scalpel under the rectangle of agar with the specimen. Helped with twicers, flipping around the agar slab, to expose the specimen on the other side (Fig. 2E, F). If the specimen is dislodged from the agar, add embryo medium to keep it under water. Gently flip it around. Flipping around specimens that have dislodged from the agar slab involves directly contacting the specimen and tends to cause more harm.
- Add 2% agarose around the rectangle of agar or over the specimen if it was dislodged (Fig. 2G, H). Caution should be used in not adding too much agar above the head of the specimen. When too deep, the needle gets deflected from its target, and it is difficult to see the structures.
- Repeat the previous steps to dislodge the second lens.

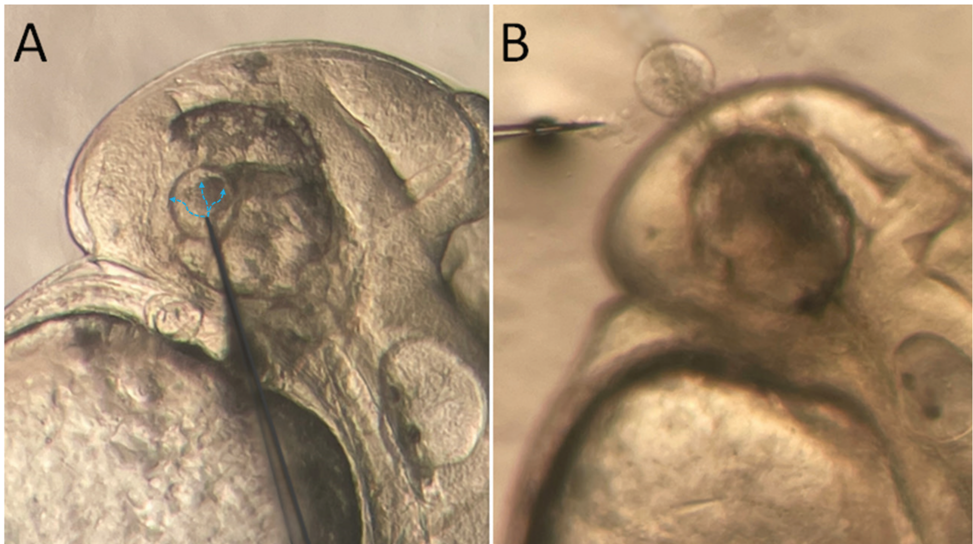

Figure 5. Style #3 for doing lens ablations **A** with the micromanipulator, slowly bring down the needle just by the side of the lens, closer than the previous two styles, and puncture the surface ectoderm. While inside the optic cup, position the needle so that it is above the lens instead of below. Gently scrape the overlaying agarose and scrape the tissue over the lens **B** the tissue will tear, and the lens will float up if there is liquid. It may need some nudging with the needle.

Style #3 works best in younger embryos that have just hatched. In older specimens, the tissue covering the lens has grown, which may require the stronger tearing of styles #1 or #2. Style #3 is the preferred style of lens removal if lenses are to be collected for transplants or other studies.

Part 5: Transplantation procedure

In this case, at the beginning of the protocol, after embedding the specimen in 2% agarose, overlay it with EM containing 1.2% agarose. Use style #3 preferentially. Once free, lenses will float in the medium. Using the blunt needle, carefully push the lens of the donor to just above where the host lens would normally be, and then push it down into the eye with the blunt needle. The host lens may be discarded.

Leave donor and host embryos in 1.2% agarose for 30–60 minutes, then release them from the agarose using the sharp needle and transfer them into EM in the incubator.

Results

Bilateral lensectomies using microinjection needles made from glass capillaries attached to a manual micromanipulator were extremely successful compared to previous results using tungsten needles held by hand (Hélène Hinaux, personal communication; Elipot et al. 2013). Two hundred fifty-six live specimens were obtained from three broods. Of them, 96 underwent lens ablations on both sides, and 160 did not, serving as control siblings. Both the experimental and the control groups were kept under the same conditions. Initial postoperative survival was 100%, as reported in Espinasa et al. (2014) and Blin et al. (2018). In the three broods used for those experiments, no single specimen died during the operation, and all were alive 24 hrs post-operation. After one week post-operation, 88 (91.6%) lensectomized fish were alive, and 158 (98.7%) control fish were alive. After one month, 80 (83.3%) lensectomized and 145 (90.6%) control fish remained. Survival rates were equivalent between lensectomized fish and their siblings on which no operation was performed and kept in the same incubator (P=0.112, Fisher exact test). No impact on non-targeted tissues was seen in both experimental and control group.

Time dedicated to conducting ablations in each brood was about 10 hrs, giving an average of about 3.3 successful double ablations per hour. All treated specimens developed normally and had equivalent body sizes to their untreated siblings. One week after the procedure, individuals on which a single side lensectomy was performed already one significantly smaller eye (Fig. 1E). The success of the lensectomy procedures in triggering eye degeneration was observed by the specimens' significantly regressed eyes compared to untreated specimens of the same brood after a few months of development (Fig. 6).

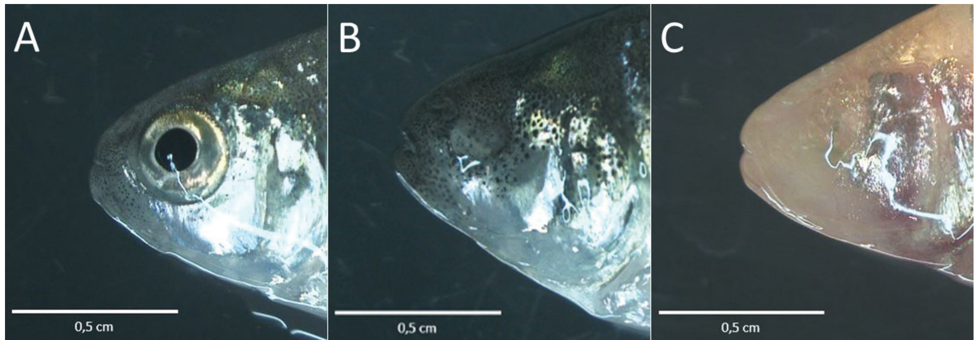

Figure 6. Four month-old *Astyanax mexicanus* **A** non-operated surface fish **B** lensectomized surface fish **C** Pachón cavefish.

Discussion

The technique for embryonic lens removal described for *Astyanax* fish constitutes a significant modification for improvement. It is also readily applicable to zebrafish. Production of healthy individuals with double lensectomies increases by at least an order of magnitude (Hélène Hinaux, personal communication). Hundreds of specimens can now be made available for study, thus solving previous sample size limitations that hindered research on the developmental effects of eye regression.

Compared with previous lens removal techniques performed on *Astyanax* or zebrafish (see video from Zhang et al. 2009, at 1:25–1:35 minutes; https://www.jove.com/fr/v/1258/lens-transplantation-zebrafish-its-application-analysis-eye?section=0&&?section=0&&), it is noticeable the reasons our technique constitutes a significant improvement. As seen in this video, previous techniques that control the needle by hand are very jittery. While the lens has a diameter of only 50 μm, hand shaking makes the needle oscillate and "jump" up to 100 μm. The eye and head of the fish embryo are seen to be pushed broadly and often. The precision for puncturing the epithelium around the lens is very difficult. Puncturing neighboring structures, such as the brain or heart, often happens with the previous technique. On the contrary, with a micromanipulator, movements of the needle can be controlled easily at less than 5 μm. Microinjection needles made from pulled glass capillaries also appear to puncture the tissues more smoothly than previously used tungsten needles.

Several steps require special attention

Healthy conditions for the breeding colony: Survivorship of embryos can be drastically different between laboratories due to the conditions in which parents and the embryos are kept. The technique described here can produce live embryos, with the limiting factor being the general survivorship of embryos within the specific laboratory conditions they are kept.

Clean needles: Throughout the procedure, the needles progressively get covered in what appears to be a fatty substance (Fig. 1D). This "glob" blunts the needle, and instead of easily puncturing the tissue, it pushes down the eye until uncontrolled rupture may happen. Sometimes the needle tip can be cleaned by immersion in the agar and sliding the needle sideways while inside the agar. Nonetheless, it is best to exchange it for a new one after about three lenses have been removed, or when the glob develops.

The agar's depth significantly affects the efficiency of the procedure (Fig. 2C). Especially when doing lens ablation on both sides, one side will be overlaid by more agar. Practice until achieving correct conditions. We found that, as a generality, adding too much agar was a more significant problem.

Gentle, slow motions are to be done with the micromanipulator throughout the process (Fig. 1C). While up to 10 fish were successfully put in a row to undergo the procedure, we found that a slower approach, working with about three fish at a time, was the most efficient (Fig. 2G–I).

Acknowledgements

This procedure follows, to a large extent, the transplantation technique developed for cavefish by the lab of Bill Jeffrey (Yamamoto and Jeffery 2002) and then modified in Espinasa et al. (2014). Support for a short sabbatical stay at the Paris-Saclay Institute to LE was provided by a VPAA grant from Marist College and by ANR (Agence Nationale pour la Recherche) grant CAVEMOM to SR. Many thanks to Krystel Saroul for taking care of our *Astyanax* colony and the operated fish. Jordi Espinasa and Monika Espinasa reviewed the manuscript.

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