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PROTOCOL

Generation of axolotl hematopoietic chimeras

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Abbreviations used: HSCs, hematopoietic stem cells; HCT, hematopoietic cell transplantation

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Abstract Wound repair is an extremely complex process that requires precise coordination between various cell types including immune cells. Unfortunately, in mammals this usually results in scar formation instead of restoration of the original fully functional tissue, otherwise known as regeneration. Various animal models like frogs and salamanders are currently being studied to determine the intracellular and intercellular pathways, controlled by gene expression, that elicit cell proliferation, differentiation, and migration of cells during regenerative healing. Now, the necessary genetic tools to map regenerative pathways are becoming available for the axolotl salamander, thus allowing comparative studies between scarring and regeneration. Here, we describe in detail three methods to produce axolotl hematopoietic cell-tagged chimeras for the study of hematopoiesis and regeneration.

Keywords: axolotl, chimera, HCT, hematopoiesis, regeneration

BACKGROUND

Hematopoietic stem cells (HSCs) and their progeny are crucial at all stages of wound healing [1–3]. Interestingly, overall regenerative capacity is inversely correlated with immune development and evolutionary advances. Anuran amphibians (frogs) display the ability to completely regrow their hind limbs and tail, but only preceding metamorphosis during which their immune system matures [4–6]. Higher mammals like humans are also able to elicit scar-free regenerative responses to dermal lesions, but solely prior to birth [7]. Among vertebrates, only urodele amphibians (salamanders) are capable of extensive tissue and organ regeneration, including limbs, throughout all stages of life [1,8,9]. A better understanding of these fully regenerative animals and their immune systems may lead to new therapeutic approaches for improved mammalian wound healing and tissue regeneration [10].

Hematopoietic cell lineage tagging by hematopoietic cell transplantation (HCT) with a reporter gene like GFP is often used in mice to more easily identify the roles of immune cells during repair, regeneration, or other inflammatory processes [11–13]. The axolotl salamander makes an excellent animal model in which GFP-tagged immune cells can be tracked in real-time due to its nearly transparent skin. In addition, the axolotl's immune system has a great deal of homology with that of higher vertebrates, including humans [14–18]. The axolotl thus provides a unique model in which hematopoiesis or immune function can be contrasted between a regenerative axolotl injury and a non-regenerative/scarification response in a mammalian model. GFP immune cell tagging facilitates subsequent analysis by FACS to characterize signaling pathways, growth factors, and various cell populations controlled by gene expression patterns.

In this protocol, we describe the adaptation of HCT to the axolotl based on common mammalian methodology but also provide details for hematopoietic cell tagging by embryo microsurgeries. Injecting cell suspensions of GFP+ liver and spleen into white lethally irradiated (950 rads) adult axolotls or non-conditioned larvae results in sustained donor-derived multi-lineage immune reconstitution. Taking advantage of the axolotl's large manipulable embryos, we bisected GFP+ embryos (stages 14-20) and fused the cephalic portion with the caudal portion of either white or nucCherryRed+ embryos. The two halves fuse and successful surgeries result in normal animals with blood cells containing GFP, nucCherryRed, or a mixture of blood cells containing one of the two fluorescent proteins.

MATERIALS

Animals

White mutant (d/d), GFP+ or nucCherryRed+ CMV:Chicken β-actin (pCAGGs-eGFP+) promoter-driven transgenic axolotls and embryos were purchased from the Ambystoma Genetic Stock Center (AGSC), or bred in house from AGSC founder animals. Animals were staged as described [19] and maintained in Holtfreter's solution. Adult axolotls were one year or older. Microinjections were performed on embryos and larvae up to 3 months of age. All animals in this study were treated humanely and all procedures were approved under the University of Florida IACUC protocol #201202645.

Reagents

- ✓ Chlorhexidine
- ✓ Sodium chloride (NaCl) (Fisher Scientific, # S640-50)
- ✓ Potassium chloride (KCl) (Fisher Scientific, # P217-3)
- ✓ Calcium chloride ($CaCl_2$) (Fisher Scientific, # C79-3kg)

- ✓ Magnesium sulfate ($MgSO_4$) (Fisher Scientific, # M63-3)
- ✓ NovAqua Plus water conditioner (Aquatic Eco-Systems, # NA64P)
- ✓ AmQuel Plus ammonia detoxifier (Aquatic Eco-Systems, # AM64P)
- ✓ L15 Media (Cellgro, # 10-045-CV)
- ✓ Distilled water
- ✓ FBS – Advantage (Atlanta Biologicals, # S11050)
- ✓ Penicillin-streptomycin solution (Invitrogen, # 15140122)
- ✓ Insulin-Transferrin-Selenium (Life Technologies, # 51300-044)
- ✓ Tricaine (Ethyl 3-aminobenzoate methanesulfonate salt) (SIGMA-ALDRICH, # A5040-250)
- ✓ Sodium bicarbonate (SIGMA-ALDRICH, # S5761-1)
- ✓ PBS (Fisher Scientific, # MT21040CMRF)
- ✓ 70% ethanol (Diluted with dH_2O) (Decon Labs, # 64-17-5)
- ✓ Sulfamerazine (SIGMA-ALDRICH, # S8876-250)
- ✓ Blue food dye (McCormick, UPC # 050428138861)
- ✓ Calcium chloride dihydrate ($CaCl{\cdot}2H_2O$) (Fisher Scientific, # C79-500)
- ✓ HEPES (Fisher Scientific, BP410-500)
- ✓ Sodium hydroxide (NaOH) (Fisher Scientific, # BP359-500)
- ✓ G418 (Cellgro, # 61-24-RG)

Equipment

- ✓ Gloves (Fisher Scientific, # 191301597D)
- ✓ Plastic transfer pipettes (Fisher Scientific, # 13-711-20)
- ✓ 35 mm cell culture dishes (Fisher Scientific, # 08-757-11YZ)
- ✓ Agarose (SIGMA-ALDRICH, # A9045-10)
- ✓ Watch maker forceps (Roboz, RS-5065)
- ✓ Blunt tipped Forceps (Roboz, RS-5130)
- ✓ Microsurgical scissors (Roboz, # RS-5603)
- ✓ Standard surgical scissors (Roboz, # RS-5910)
- ✓ Irradiator (137 Cesium source irradiator)
- ✓ Microinjector (Leica mechanical micromanipulator)
- ✓ Kwik-Fil Borosilicate Glass Capillaries (1 mm outer diameter, no filament; World Precision Instruments, # MTW100F-4)
- ✓ Needle puller (Sutter Instrument Co, # P-97)
- ✓ Dissecting stereo microscope (Leica, # MZ6)
- ✓ Scalpel or razor (Personna, # 94-120-71)
- ✓ Insulin syringe (Excel International, # 26027)
- ✓ 6-0 nonabsorbable monofilament polypropylene sutures (Ethicon, # 8706)
- ✓ 4-0 nonabsorbable monofilament polypropylene sutures (Ethicon, # 8831H)
- ✓ Fully frosted microscope slides (Fisher Scientific, # 12-544-5CY)
- ✓ 70 µm nylon cell strainer (Fisher Scientific, # 22-363-548)
- ✓ Bench top centrifuge (Sorvall, # RT7 Plus)
- ✓ 50 ml conical centrifuge tubes (Homecare Supplies, # 352070)
- ✓ Non-toxic reusable adhesive Handi-tak (Super Glue Corporation, # HT2)
- ✓ Hemocytometer (Reichert Bright-Line, # 1483)
- ✓ Vacuum driven filtration system (Fisher Scientific, # SCGPU05RE)
- ✓ Parafilm (Fisher Scientific, # 1337412)
- ✓ Hot bead sterilizer (Fine science tools, # 18000-45)

Recipes

- ✓ 40% Holtfreter's solution (167 L / 44 gal)
  - 5 g /1 teaspoon KCl
  - 12.5 g /2.5 teaspoons $CaCl_2$
  - 30 g / 2 tablespoons $MgSO_4.7H_2O$
  - 237 g / 1 cup NaCl
- ✓ Steinberg's solution (1 L)
  - 3.4 g NaCl
  - 0.05 g KCl
  - 0.03 g $CaCl{\cdot}2H_2O$
  - 1.1 g HEPES
  - 1x antibiotic-antimycotic
  - 25 mg G418
  - pH to 7.4 with NaOH
  - Filter sterilize
- ✓ APBS
  - Dilute PBS to 70% with dH_2O
- ✓ Axolotl fibroblast media
  - 60% L-15 media with distilled water
  - 5% FBS
  - 1x penicillin-streptomycin
  - 1x insulin-transferrin
- ✓ Tricaine methanesulfonate solution (0.1%)
  - 1.5 L dH_2O
  - 1.5 g Tricaine methanesulfonate
  - pH to 7.4 with sodium bicarbonate

PROCEDURE

As a general note: axolotl cells require a different osmotic pressure than mammalian cells thus requiring standard buffers and media be diluted to 70% normal strength with distilled water prior to use.

Ablative hematopoietic cell transplantation

This procedure is for HCT from a young (3 month old) GFP+/nucCherryRed+ larval axolotl into a white adult (1 year or older) recipient axolotl that will not result in graft-versus-host disease (GVHD). If the effects of GVHD are a desired component of the study, it can be elicited by using an adult donor and an adult recipient.

1. Irradiation of recipient axolotls should be done 1-2 days prior to HCT:
   - 1.1. Anesthetize white adult recipient axolotl by submersion in 0.1% tricaine solution until unresponsive (10-20 min depending on size).
   - 1.2. Place the axolotl in a container suitable for the irradiator with its tank water (40% Holtfreter's solution) and protect the animal's head and gills with lead shielding (**Fig. 1A**).

1.3. Irradiate axolotl with 950 rads and return the animal to its housing tank for recovery.

2. Harvesting of hematopoietic organs (liver and spleen) with non-survival surgery of donor to maximize number of hematopoietic cells. (Refer to step 3 for survival surgery procedure):

2.1. Sterilize blunt-nosed forceps, surgical scissors, and any other surgical tools in 70% ethanol, hot bead sterilizer, or autoclave.

2.2. Anesthetize GFP+/nucCherryRed+ larval donor axolotl by submerging in 0.1% tricaine solution for 5-10 min.

CAUTION: Time to anesthetization of animals can vary. The animal should be closely monitored to ensure total anesthetization before proceeding.

2.3. Remove axolotl from the tricaine solution and place on dissecting tray or moist paper towel.

2.4. With a scalpel/razor or surgical scissors decapitate the anesthetized animal.

2.5. With surgical scissors cut the ventral skin and muscle tissue starting from the neck opening towards the tail to expose the ventral cavity.

2.6. Remove the entire liver (large light pink-yellow or grey organ at the top of the ventral cavity) and spleen (dark red organ attached to the stomach) by gently lifting organs with blunt-nosed forceps and cut away connective tissue with surgical scissors (**Fig. 1B**).

2.7. Locate and remove the gall bladder from the liver.

2.8. Place the liver and spleen in ice cold APBS.

3. Harvesting of hematopoietic organs (liver and spleen) with survival surgery of donor for further studies:

3.1. Sterilize watchmaker forceps, microsurgical scissors, and any other surgical tools in 70% ethanol, hot bead sterilizer, or autoclave.

3.2. Anesthetize GFP+/nucCherryRed+ larval donor axolotl by submerging in 0.1% tricaine solution for 3-5 min or about 2 additional min after the animal is completely unresponsive.

CAUTION: Tricaine dose should be scaled for size to prevent anesthetic death. This can be done by lowering the concentration of tricaine or reducing exposure time. For this procedure we maintain the concentration but reduce exposure time for larvae. Variability between animals can occur and thus the animal should be closely monitored to determine the level of anesthetization.

3.3. Transfer anesthetized axolotl to 40% Holtfreter's solution with 0.5% sulfamerazine for 30 s.

3.4. Place animal on dissecting tray or moist paper towel under a dissecting microscope and make sure that the animal's skin does not dry out throughout the procedure by dripping water on it periodically.

3.5. With a scalpel/razor make two small incisions in the skin directly over the liver and spleen just enough to puncture the skin. This will be visible under the dissecting microscope (**Fig. 1C**).

3.6. With microsurgical scissors increase the size of the incisions to the minimum required length for access to the liver and spleen.

3.7. Using watchmaker forceps pull the caudal tip of the spleen through the incision in the skin and cut approximately half of the spleen with microsurgical scissors.

CAUTION: Care must be taken not to pull the spleen too far from the stomach or it could detach entirely.

3.8. Using watchmaker forceps pull one of the left or right edges of the liver through the incision in the skin and cut off approximately 1/3 of the liver.

CAUTION: The gall bladder and intestines are attached to the lower portion of the liver so care must be taken not to damage these tissues.

3.9. Place the pieces of liver and spleen in ice cold APBS.

3.10. Make sure the liver and spleen of the animal are not jutting out from the skin.

3.11. Place animal back in 40% Holtfreter's solution with 0.5% sulfamerazine.

NOTE: Larvae should have minimal bleeding if any and do not need sutures or dermal adhesives for the incisions in the skin. These animals will completely heal within 2-3 weeks if kept in clean water with sulfamerazine.

4. Grind the liver and spleen separately in to two 35 mm petri dishes containing 1 ml of APBS using two fully frosted glass slides.

TIP: Cells will flow into the APBS if the bottom edges of the glass slides are touching the APBS while grinding tissues.

5. Keep liver and spleen cell solutions separate and pass them through 70 µm nylon cell strainers directly into two 50 ml conical centrifuge tubes to make single cell solutions.
6. Count cells with a hemocytometer.
7. Spin cells down in a centrifuge at approximately 244g for 5 min.
8. Remove supernatant and resuspend cells at least at 5×10^5 cells per 150 µl of APBS for each organ then place on ice.
9. Anesthetize white adult recipient axolotl by submersion in 0.1% tricaine solution until unresponsive (10-20 min depending on size)
10. Dilute blue food dye with APBS and add one drop of dilute solution to liver and spleen cell solutions.
11. Load at least 175 µl of each cell solution into one insulin syringe (minimum total of 350 µl).
12. Locate the hind leg vein on the recipient axolotl and inject cell solution intravenously near the point where the leg joins the body avoiding the injection of any air (**Fig. 1**).

    NOTE: If the syringe plunger is not easily moving, then the needle tip is likely in the muscle and not in the vein. The cell solution should also be seen flowing through blood vessels all around the injection site.
13. As soon as the syringe is removed, swing the leg back towards the tail to block the injection site with the tail and apply light pressure for 5-10 s (prevents leakage of cell solution from the leg).
14. Return recipient axolotl to housing tank with clean 40% Holtfreter's solution.
15. Engraftment will be evident by observing donor-derived blood cells in the skin or circulation within 1 month. If an adult donor was used, signs of GVHD will be noticeable in the recipient between 6-8 weeks.

Microinjection hematopoietic cell transplant (non-ablative)

This procedure is for HCT from an adult (1-2 year old) GFP+/nucCherryRed+ larval axolotl into white embryos or larvae (stages 25 of development through 3 months of age) recipient axolotls that will not result in GVHD. No chemical or irradiative conditioning is necessary.

16. Make glass pulled needles using borosilicate glass capillaries and needle puller.
17. Break the tip of the glass pulled needles using watchmaker forceps so that they can be loaded.
18. Prepare a 35 mm petri dish with reusable adhesive and make a depression in which the embryo or larvae will fit as seen in **Figure 2**.
19. Mix fresh Steinberg's solution with fresh tricaine solution at a ratio of 3:1 and fill the petri dish with this solution. Cover it when not in use.
20. Prepare glass pulled needles and microinjection apparatus.
21. Harvesting of hematopoietic organs (liver and spleen) with survival surgery of donor for further studies:

    21.1. Anesthetize GFP+/nucCherryRed+ adult donor axolotl by submerging in 0.1% tricaine solution for 10-20 min or for an additional 2-5 min after the animal is completely unresponsive.

    CAUTION: Time to anesthetization of animals can vary thus the animal should be closely monitored to ensure total anesthetization before proceeding.

    21.2. Transfer anesthetized axolotl to 40% Holtfreter's solution with 0.5% sulfamerazine for 30 s.

    21.3. Place animal on dissecting tray with a moist paper towel and make sure that the animal's skin does not dry out throughout the procedure by dripping water on it periodically.

    21.4. Apply 0.75% chlorhexidine solution with sterile gauze to the areas of the skin over the spleen and liver.

    21.5. With a scalpel/razor make two small incisions in the skin directly over the liver and caudal half of the spleen just enough to puncture the skin and muscle where the internal organs will be visible (**Fig. 1B**-C).

    21.6. Tip: the outline of the spleen will be lightly visible due to its dark red color on the left side of the axolotl's body. Make the incisions parallel to the direction of the body to allow adjustments in size to be made if necessary.

    21.7. With surgical scissors increase the size of the incisions to the minimum required length for access to the liver and spleen (less than 1 inch in length for an adult axolotl).

CAUTION: if the spleen is cut there will be a large amount of bleeding making it difficult to distinguish the spleen from blood or chunks of coagulating blood.

21.8. Using blunt-nosed forceps pull the caudal tip of the spleen through the incision in the skin and cut approximately 1/4 - 1/2 of the spleen with surgical scissors.

NOTE: Microinjections do not require many cells and thus 1/4 to 1/3 of an adult spleen should provide enough cells for at least 20 transplantations.

21.9. Place the piece of spleen in ice cold APBS.

21.10. Push the spleen back under the skin and use a simple interrupted suture pattern on the skin with a 4-0 or 6-0 nylon monofilament.

21.11. Using blunt-nosed forceps pull one of the left or right edges of the liver through the incision in the skin and cut off approximately 1/4 - 1/3 of the liver.

CAUTION: The gall bladder and intestines are attached to the lower portion of the liver so care must be taken not to damage these tissues.

21.12. Place the piece of liver in ice cold APBS.

21.13. Push the liver back under the skin and use a simple interrupted suture pattern on the skin with a 4-0 or 6-0 nylon monofilament.

21.14. Place animal back in clean 40% Holtfreter's solution with 0.5% sulfamerazine. Keep animals in clean water and with the sulfamerazine for about 3 weeks.

22. Grind the liver and spleen separately in to two 35-60 mm petri dishes containing 1-5 ml of APBS using two fully frosted glass slides.

TIP: Cells will flow into the APBS if the bottom edges of the glass slides are touching the APBS while grinding tissues.

23. Keep liver and spleen cell solutions separate and pass them through 70 µm nylon cell strainers directly into two 50 ml conical centrifuge tubes to make single cell solutions.

24. Spin cells down in a centrifuge at approximately 244 g for 5 min.

25. Remove most of the supernatant and resuspend spleen cells in leftover supernatant.

26. Remove supernatant in liver cells and aspirate off white adispose cells that overlay hematopoietic cells. Then resuspend liver cells in a small amount of APBS to maintain a highly concentrated but fluid cell solution.

27. Cell solutions can be mixed or kept separate but should be maintained on ice. A small amount of food dye can be added to help visualize the infusion of the cell solution into the heart.

28. Place embryo or larva into the 35 mm petri dish with Steinberg's and tricaine solution.

29. With a Pasteur pipette mix cell solution and dispense one drop onto clean parafilm, petri dish, or plastic lid.

30. Load about 1/3 of the glass microinjection needle with cell solution.

CAUTION: Cells will settle to the bottom of the drop rapidly so the glass needle needs to be loaded quickly or loaded with the bottom of the drop.

31. Ensure that the embryo or larvae is anesthetized and position its back into the depression made in the adhesive (ventral side up) as seen in **Figure 2**.

32. Position the glass needle at an approximately 60° angle over the heart and lower it into the water.

CAUTION: Some of the Steinberg's and tricaine solution will move into the syringe.

33. Under a dissecting microscope begin to expel some of the cells to remove the Steinberg's and tricaine solution that moved into the glass needle.

34. As cells begin to flow out of the needle, lower it into the larva's or embryo's heart and make sure it punctures it but does not go through it.

35. Inject the cell solution slowly until it is clearly all throughout the body or leaking into the ventral cavity.

NOTE: Not injecting enough cells will decrease the engraftment success of this procedure but causing too much internal damage to the animal will reduce survival. The heart may also stop but will restart once the needle has been removed and pressure released.

36. Remove the needle from the heart while holding the animal gently down with forceps.

37. Transfer the animal into freshly made Steinberg's solution. Keep animals in this solution for 24 h and then transfer them into clean 40% Holtfreter's solution.
38. If engraftment of long-term HSCs is unsuccessful, donor cells will disappear within 6-10 weeks.

Embryo fusions

This procedure results in the generation of one animal made up of two genetically different halves. Blood cells will be of the same color as the spleen in the adult animal and can be tracked in the end of the body of the other color. If done correctly, animals will develop normally.

39. Prepare 35 mm petri dishes by pouring autoclaved 2% agarose to approximately 1/3 full and allow to solidify.
40. Sterilize watchmaker forceps and microsurgical scissors in 70% ethanol or hot bead sterilizer.
41. Under a dissecting microscope, identify and collect embryos at stages 14–20.
42. Under a dissecting microscope, manually de-jelly the embryos using two watchmaker forceps carefully so as not to damage the embryo.

    TIP: Make one initial puncture with the point of one of the forceps while holding it steady with the other forceps. Then, put one end of the stabilizing forceps into the hole made. With both forceps in the jelly capsule, tease it apart slowly until the embryo falls out.
43. Transfer embryos using appropriately sized Pasteur pipettes into fresh 100% Steinberg's solution for 5 min as a first wash. Wash two more times in separate containers for at least 5 min each time.

    CAUTION: If the embryos touch the surface of the water, the surface tension will tear them apart.
44. Fill agarose lined dishes with 100% Steinberg's solution and transfer in two same staged embryos of different colors.
45. Under the dissecting microscope, make two small depressions in the agarose using watchmaker forceps that will precisely fit each embryo (**Fig. 3**).
46. Transversely cut each embryo in half with microsurgical scissors ensuring that the cut is at the same location on each embryo (**Fig. 3B**-C).

    CAUTION: If the embryos touch the surface of the water, the surface tension will tear them apart.
47. Remove clear embryo membrane carefully with forceps.
48. Pair the anterior end of one embryo with the posterior end of the other (**Fig. 3B**-C).
49. Move paired halves into depressions made in agar with neural folds touching and cover dishes with lids. Intimate opposition of the neural folds is essential for proper healing and further development (**Fig. 3B**-C).
50. Move dishes very carefully to the side where they need to remain undisturbed for about 96 h. Embryos will fuse entirely within 24-48 h at 20°C (**Fig. 3C**).
51. Transfer embryos into fresh 100% Steinberg's solution for another 7 days in covered petri dishes.
52. Finally, transfer embryos to 40% Holtfreter's solution. Many embryos will have defects due to improper healing and will die.

ANTICIPATED RESULTS

The protocols described here will produce hematopoietic chimeric axolotls with fluorescent protein-tagged blood (**Fig. 4-8**). We have previously published extensive results on these methodologies in the journal Blood [14].

Ablative HCT

Resultant animals will display engraftment and donor-derived cells in the skin and circulation within 2-4 weeks. By 2 months significant levels of these cells will be present. If the donor animal was an adult, GVHD or infections will be visible by 6-8 weeks. The success rate of this procedure should be 80% or greater (**Fig. 4**).

Microinjection HCT

Microinjected embryos and larvae will have visible donor-derived cells within 1-3 days after the procedure. If engraftment of long-term HSCs is unsuccessful, quantities of donor-derived cells will be seen decreasing in the skin and circulation at about 2 months after the procedure. We have achieved a 90% success rate with this technique (**Fig. 5**).

Embryo fusions

Embryos should fuse entirely within 24-48 h but may need additional healing time in some cases. We have had about a 1-2% success rate of rearing these embryonically fused animals to at least 6 months of age but the majority that reach this age continue to live for years. Blood can be seen circulating once the gills begin to develop and heart begins to beat. Blood of two colors will usually be mixed early in development, but depending on the location of the cut, can shift heavily towards one of the two within 7 months. If a white axolotl embryo is used and the spleen develops as white, all or nearly all fluorescent blood will convert to white and not fluoresce. The nucCherryRed+ cells are more difficult to see since the fluorescent protein is restricted to the nucleus, unlike the GFP that is spread throughout the cytoplasm. Cell morphology is also more easily visualized in GFP+ cells (**Fig. 6-7**).

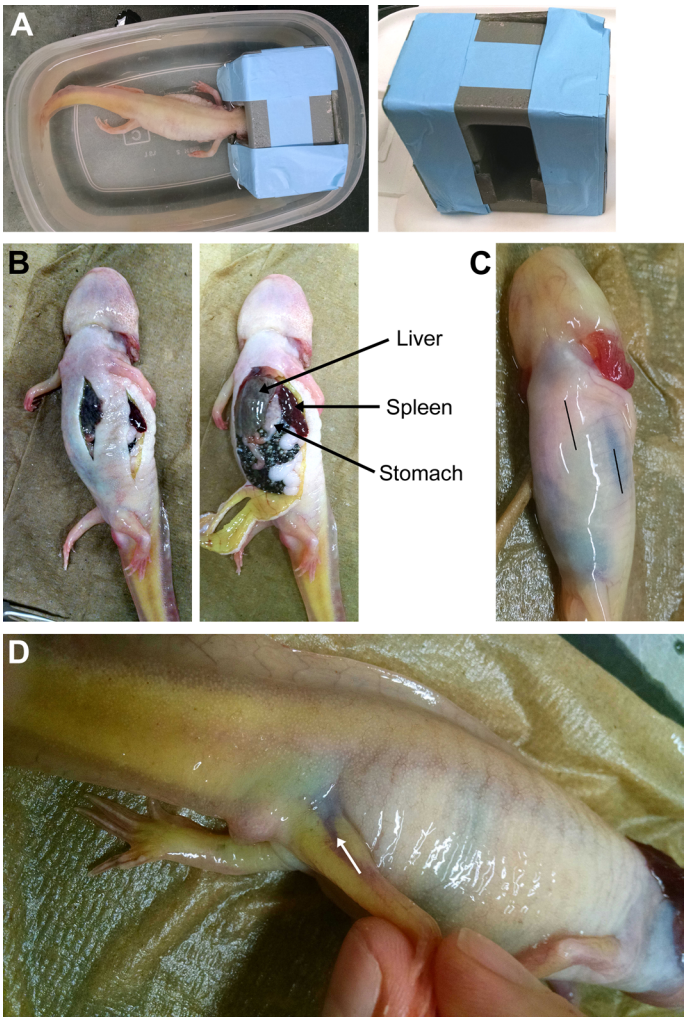

Figure 1. Three main steps in irradiative HCT: irradiation setup, anatomy for cell harvesting, and recipient injection site. A. Lead block keeps the axolotl's head and gills protected from irradiation that will come from above and below the animal. Any other lead shielding that will protect the head and gills but leave the liver and spleen exposed is acceptable. **B.** Relevant axolotl anatomy of an adult and location where incisions should be made on the donor animal. Incisions are longer than necessary in this picture and should be made only as long as is necessary to harvest desired tissues if performing the survival surgery. **C.** A juvenile axolotl with black lines indicating where incisions should be made. In young animals like this one the organs are visible through the skin facilitating this procedure. **D.** HCT injection site in the recipient's leg vein. Syringe should follow the path of the arrow and only needs to puncture the vein with the tip.

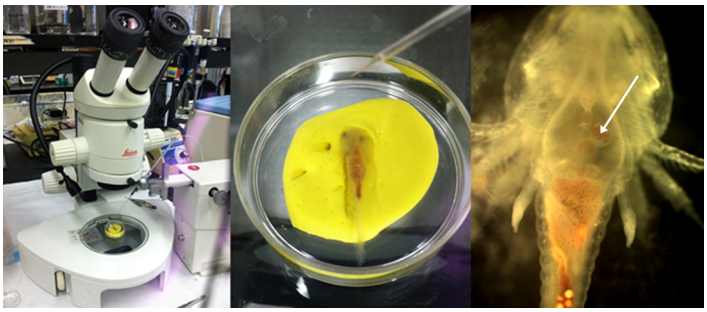

Figure 2. Microinjection setup. White arrow indicates the heart.

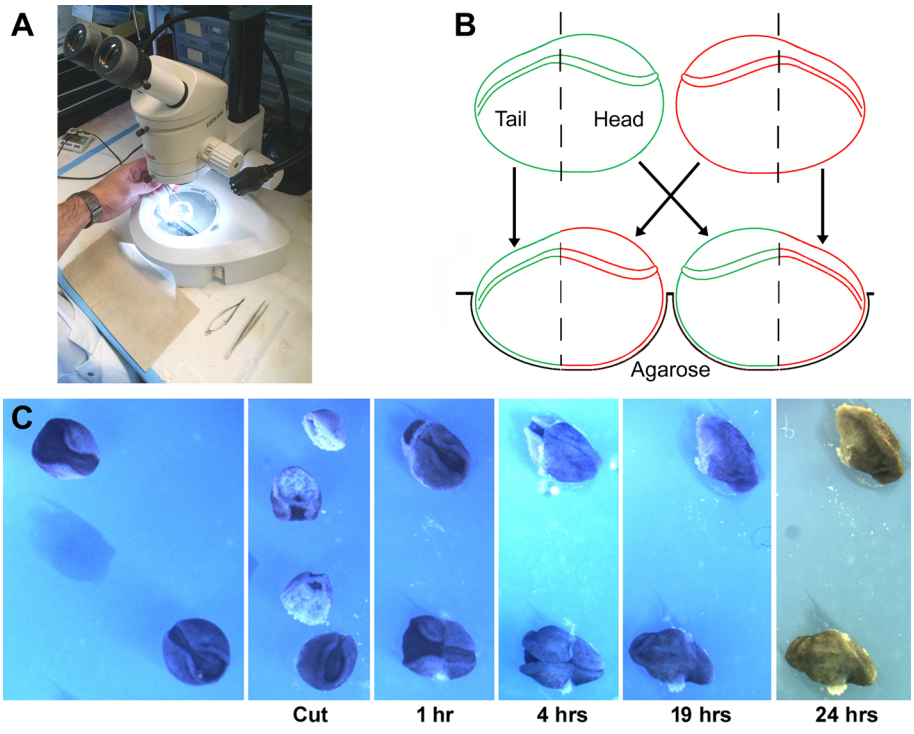

Figure 3. Embryo fusion procedure in agarose lined dishes as seen under a dissecting microscope. A. Dissecting microscope setup. **B.** Side view diagram of the embryo surgical procedure. **C.** Microscope top-down view of the embryo procedure and healing process up to 24 hrs following the procedure.

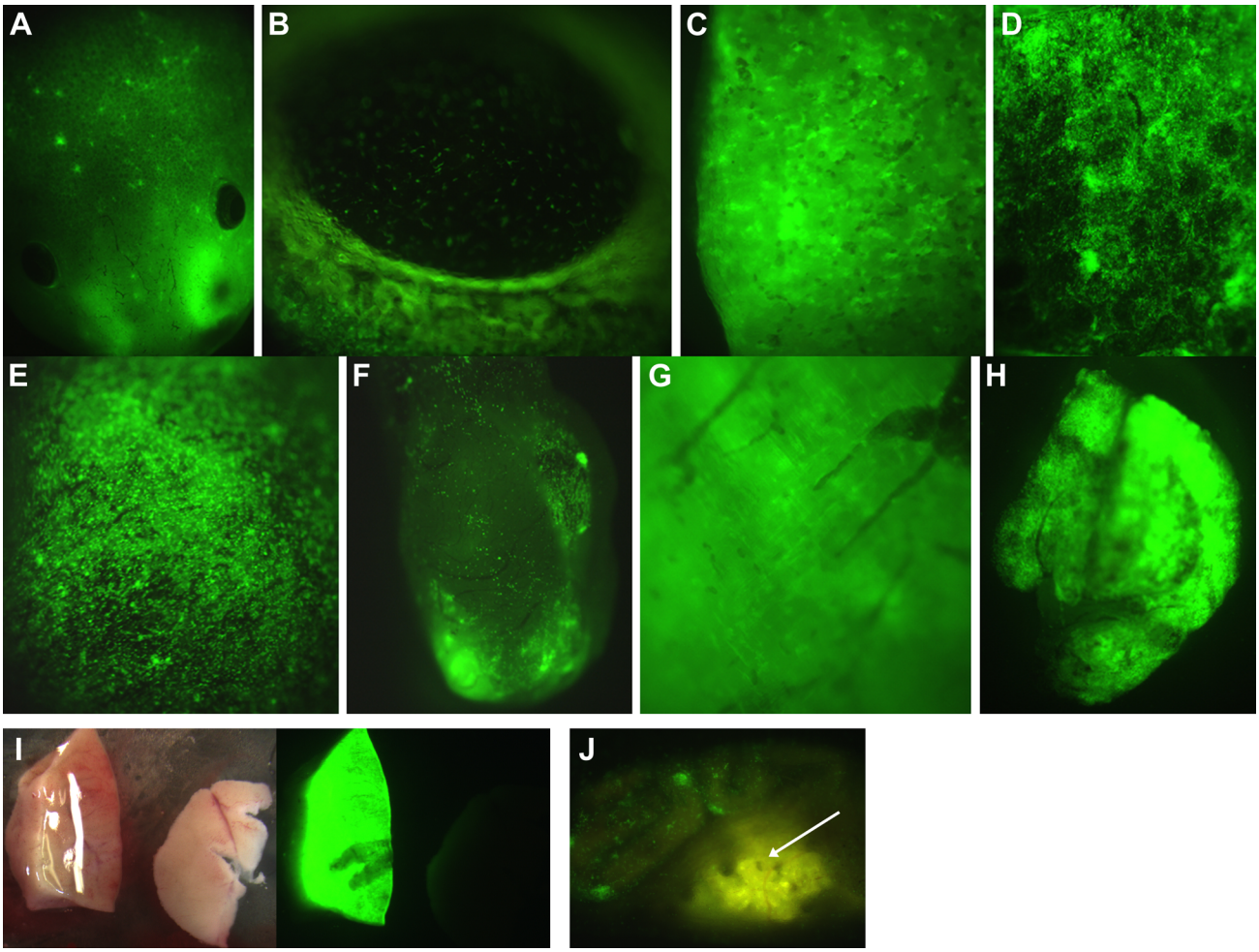

Figure 4. White adult axolotl that underwent ablative HCT from spleen and liver (imaged 4 months after HCT). A. Head. **B.** Eye. **C.** Liver. **D.** Lung. **E.** Heart. **F.** Brain. **G.** Intestine. **H.** Spleen. **I.** The liver, gastrointestinal tract, and iris of the eye are highly autofluorescent tissues in the axolotl making it difficult to discern between individual donor and host cells as seen in (B), (C), and (G). A GFP+ liver is placed next to a white liver to show that autofluorescence of non-GFP tissues is usually minimal in comparison to GFP+ tissues when seen from afar. **J.** Abdominal area of a microinjected animal demonstrating high autofluorescence. The white arrow points to the stomach with autofluorescent food. The difference in color through the GFP channel, GFP and RFP dual channel, and RFP channel alone can be useful to distinguish between true GFP fluorescence (green and will only fluoresce in the GFP channel) and autofluorescence (yellowish but will fluoresce in multiple channels). This same technique is applicable to nucCherryRed+ cells and tissues.

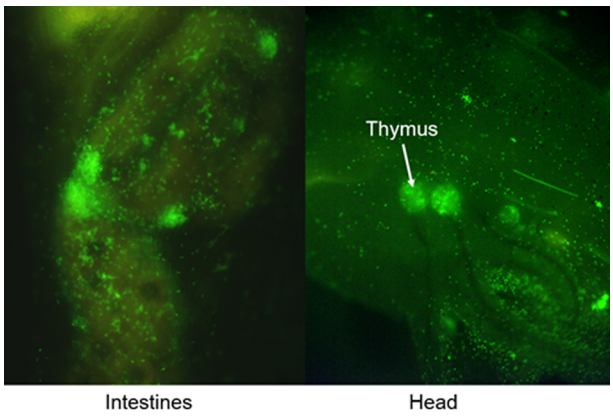

Figure 5. White larval axolotl that underwent non-ablative microinjection HCT.

TROUBLESHOOTING

Ablative HCT

If the ablative HCT procedure is not resulting in stable engraftment, increase the number of cells transplanted and use recipient animals of 1-2 years of age for best results.

Irradiated axolotls often stop feeding around 1-2 months post irradiation. If this occurs, change food to liver or live blood worms. Force feeding may be required in some extreme cases and can be performed by liquefying food pellets, worms, or liver and then transferred directly into the stomach by a syringe and appropriately sized soft plastic tube that will fit down the throat.

Infections should be treated with G418, Enrofloxacin, or other wide spectrum antibiotics. Intraperitoneal injections and other methods can be performed as described at http://www.caudata.org/cc/articles/illness.shtml [20]. We have also found some success with adding antibiotics at concentrations of 70 µg/ml to the water and monitoring recovery.

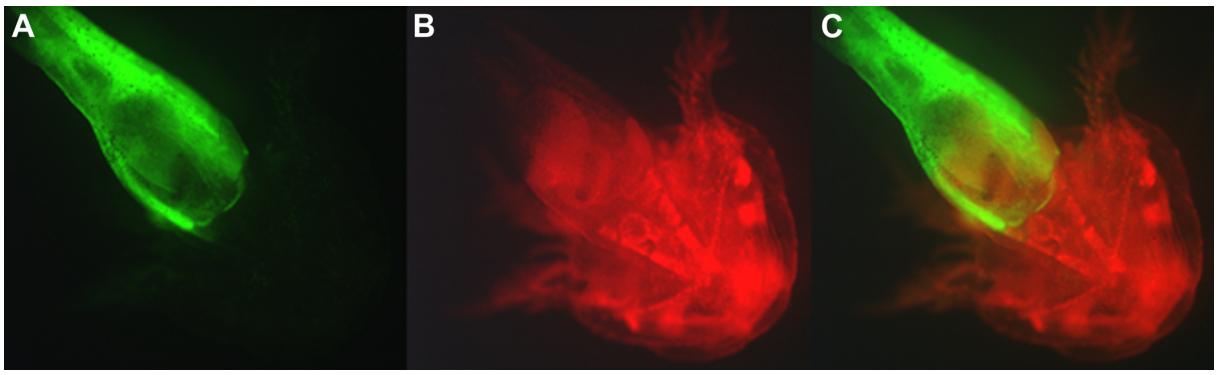

Figure 6. Ventral side of a larval axolotl made through the fusion of GFP+ and nucCherryRed+ embryos. A. Green channel. **B.** Red channel. **C.** Green and red dual channel.

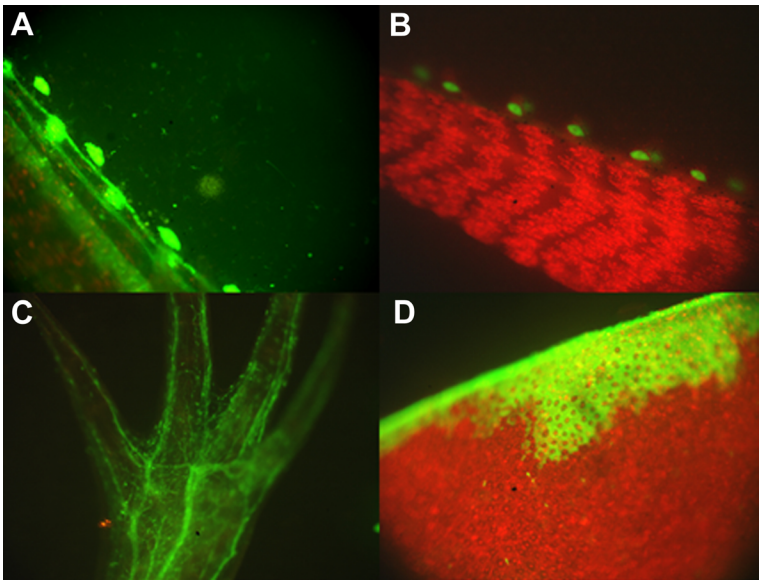

Figure 7. Another larval axolotl derived through embryonic fusion. A. Tail seen in the green channel. **B.** Tail seen in the green and red dual channel. **C.** Hand seen in the green channel. **D.** Tail fin seen in the green and red dual channel.

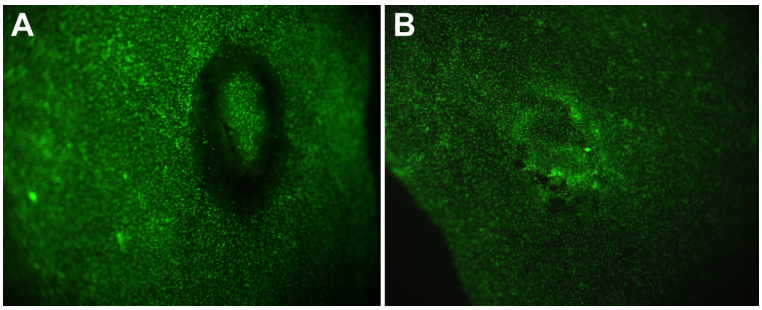

Figure 8. In vivo GFP+ immune cell recruitment to the site of injury in the ablative HCT axolotl model. A. Circular cut made with a 2 mm biopsy punch razor on the tail fin. Skin was cut but left in place. **B.** The same cut at 24 h demonstrating immune cell participation in wound healing.

Microinjection HCT

Microinjections of whole liver and spleen cells can sometimes clog glass needles necessitating the tip to be manually cleaned with watchmaker forceps.

Liver and spleen tissue demonstrate minimal clotting, but EDTA at a final concentration of 10 mM in APBS will prevent clotting entirely and may facilitate microinjections.

If survival is low the week after the microinjection procedure make sure the Steinberg's media the embryos are transferred into is fresh. Maintaining the animals in filter sterilized 40% Holtfreter's solution for an additional week may also increase survival.

If engraftment rate in microinjected animals is low, increase the number of cells injected or use younger donor animals (< 2 years of age).

Embryo fusions

Embryo disintegration is indicative of bacterial or fungal infection. Ensure petri dishes are covered when not in use, solutions are freshly made, tools are properly sterilized, and embryos are washed well prior to surgery. It is also important that embryos are allowed to heal undisturbed for at least 4 days after surgery.

LIMITATIONS

Ablative HCT

Animals that undergo ablative transplants, typically live only 3-6 months depending on the age of the animal. Axolotls will have decreased appetite or stop eating entirely. Transplantation along with malnutrition results in a weakened immunity and opportunistic pathogens will cause infections. We believe death of these animals is primarily due to deterioration of the intestines.

The advantages of this technique are that it has a very high success rate and animals can be generated within 1-2 months with high donor cell engraftment.

Microinjection HCT

Microinjected animals will accept donor cells, but since no conditioning is given prior to transplant native recipient cells will also be present. These animals will display mixed chimerism and much lower levels of donor-derived cells than animals resulting from the use of the ablative HCT or embryo fusion techniques.

However, chimerism levels are high enough for donor cells to be clearly present at abundant quantities all throughout the body and in circulation. All tissues are also clearly distinguishable from blood cells and these axolotls live full natural lives.

Embryo fusions

Embryo fusions have an extremely low survival rate of 1-2% up to 6 months in our hands. Chimeras will have blood of one or two colors at varying proportions. Depending on the study, some organs may be the same color as the blood and thus immune cells will be indistinguishable from those tissues. Since this procedure has to be performed at the embryonic stage, full grown chimeras will require 1 year or more to fully mature.

This method, though difficult and lengthy, results in unique animals that can be used for a variety of developmental or cell tracking studies. Additionally, no conditioning is necessary and axolotls with successful surgeries live full normal lives.

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