

Video Article

In vivo Electroporation of Morpholinos into the Regenerating Adult Zebrafish Tail Fin

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Abstract

Certain species of urodeles and teleost fish can regenerate their tissues. Zebrafish have become a widely used model to study the spontaneous regeneration of adult tissues, such as the heart¹, retina², spinal cord³, optic nerve⁴, sensory hair cells⁵, and fins⁶.

The zebrafish fin is a relatively simple appendage that is easily manipulated to study multiple stages in epimorphic regeneration. Classically, fin regeneration was characterized by three distinct stages: wound healing, blastema formation, and fin outgrowth. After amputating part of the fin, the surrounding epithelium proliferates and migrates over the wound. At 33 °C, this process occurs within six hours post-amputation (hpa, **Figure 1B**)^{6,7}. Next, underlying cells from different lineages (ex. bone, blood, glia, fibroblast) re-enter the cell cycle to form a proliferative blastema, while the overlying epidermis continues to proliferate (**Figure 1D**)⁸. Outgrowth occurs as cells proximal to the blastema re-differentiate into their respective lineages to form new tissue (**Figure 1E**)⁹. Depending on the level of the amputation, full regeneration is completed in a week to a month.

The expression of a large number of gene families, including *wnt*, *hox*, *fgf*, *msx*, *retinoic acid*, *shh*, *notch*, *bmp*, and *activin-betaA* genes, is up-regulated during specific stages of fin regeneration⁹⁻¹⁶. However, the roles of these genes and their encoded proteins during regeneration have been difficult to assess, unless a specific inhibitor for the protein exists¹³, a temperature-sensitive mutant exists or a transgenic animal (either overexpressing the wild-type protein or a dominant-negative protein) was generated^{7,12}. We developed a reverse genetic technique to quickly and easily test the function of any gene during fin regeneration.

Morpholino oligonucleotides are widely used to study loss of specific proteins during zebrafish, *Xenopus*, chick, and mouse development¹⁷⁻¹⁹. Morpholinos basepair with a complementary RNA sequence to either block pre-mRNA splicing or mRNA translation. We describe a method to efficiently introduce fluorescein-tagged antisense morpholinos into regenerating zebrafish fins to knockdown expression of the target protein. The morpholino is micro-injected into each blastema of the regenerating zebrafish tail fin and electroporated into the surrounding cells. Fluorescein provides the charge to electroporate the morpholino and to visualize the morpholino in the fin tissue.

This protocol permits conditional protein knockdown to examine the role of specific proteins during regenerative fin outgrowth. In the Discussion, we describe how this approach can be adapted to study the role of specific proteins during wound healing or blastema formation, as well as a potential marker of cell migration during blastema formation.

Video Link

The video component of this article can be found at <http://www.jove.com/video/3632/>

Protocol

1. Resuspend Morpholino

1. Dilute 300 nM of fluorescein-tagged morpholino into 100 µl of nuclease-free water to make an approximately 3 mM solution. The morpholino solution is aliquoted into multiple paraffin-sealed microcentrifuge tubes and stored at room temperature and away from light.
2. To determine the exact morpholino concentration, dilute 5 µl of morpholino solution (or water as a blank) in 95 µl of 0.1 N HCl. Set a baseline on a spectrophotometer at 265 nm with the water blank and then determine the reading of the diluted morpholino solution. Multiply the morpholino absorbance by its determined constant and by the dilution factor to determine the concentration in ng/µl. The morpholino constant is calculated as:
Morpholino constant = molecular weight of the morpholino X 1000/molar absorbance.

The molecular weight and molar absorbance for the morpholino can be found on the "Oligo Properties" sheet provided with the product. Divide the morpholino concentration, in ng/ μ l, by the molecular weight to determine the concentration in mM. Dilute the morpholino, if necessary, to a working concentration, typically 1.2 mM.

2. Fin Amputation

1. Anesthetize adult zebrafish in either Tricaine or 2-phenoxyethanol at 1.0 mg/ml in tank water.
2. Amputate the fin using a sterile scalpel or razor blade proximal to the first lepidotrichial branching point. This should be done at the same proximal/distal location on each animal (e.g. 7 bony segments distal from the fin girdle). **IMPORTANT:** make sure to cut perfectly perpendicular to the anterior/posterior plane of the animal. Angled cuts will result in uneven fin outgrowth of the dorsal and ventral halves of the fin.
3. Return the fish to a tank. We typically maintain the fish at 33 °C to increase the regeneration rate. Depending on the experimental design, wait 0-2 days post amputation (dpa) for fin regeneration to begin before introducing the morpholino. Alternate approaches are discussed in the Discussion, below.

3. Morpholino Injection

1. The day prior to injecting the morpholino, make an injection plate (**Figure 2**). Make sure to cut out a notch at one end of the well, which helps stabilize the fish for the microinjection procedure.
2. At 2 dpa, prepare the micro-injection apparatus and morpholino solution.
3. Dilute the fluorescein-tagged morpholino to the proper concentration (recommend starting with 1.2 mM) and place in a 65 °C water bath, for 5 minutes.
4. Pull the glass needle for the micro-injection using a needle puller.
5. Load the needle with the morpholino (note: depending on whether you back-fill or front-load your needle will determine whether you load the needle first, or cut the tip first).
6. Cut the tip off the needle at an angle.
7. Follow the directions of your micro-injection system to inject an approximately 5 nl bubble of morpholino per injection. Larger volumes of morpholino could disrupt the tissue.
8. Anesthetize the fish and place on injection plate. Remove all excess liquid and orient the needle to the appropriate location, just distal to the bony ray (**Figure 3**). Using a microscope and microinjection apparatus appropriate for morpholino injections (see Table of Specific Reagents), inject the morpholino into the fin from anterior to posterior, as shown in **Figure 3**. Injecting from posterior to anterior causes the fin tissue to roll up during the injection and is very difficult.
9. Insert the needle gently into the regenerative tissue, just distal to each bony ray (marked "1" in **Figure 3**) and push distally until located in the blastema (marked "2" in **Figure 3**). Be careful not to push the needle through the entire fin. When the needle is correctly localized, inject the morpholino (i.e. click one time). At 1.2 mM, the fluorescein-tagged morpholino solution appears yellow-green, even in normal light conditions. With each injection, a yellow "puff" of morpholino solution can be visualized, which helps localize the injection to the blastema. Approximately 75 nl (10-15 clicks) of morpholino solution should be injected per bony ray. Pause ~ 1 sec between clicks. We only inject the dorsal side, using the ventral as electroporation-only control. Alternatively, one could repeat this entire procedure using a control morpholino on the ventral half of the fin.

4. Electroporation of the Morpholino

1. Immediately following injection of the morpholino, remove the injection plate from the micro-injection apparatus. Fill the injection plate well with the anesthesia solution until the fish is submerged. The electroporation is performed under the water line.
2. A 3 mm diameter platinum plate tweezer electrode (CUY 650-P3 Tweezers, Protech International) localizes the pulses to approximately one-half of the fin. Be careful not to touch the electrodes to the fin tissue. To ensure that the electrodes do not touch the fin tissue, rotate the fish on its dorsal side and look straight down the midline for the electroporation.
3. Electroporate both the dorsal and ventral (to control for a non-specific electroporation effects) sides of the fin using a CUY21 Square Wave Electroporator (Protech International, Inc.). Wipe the electrodes with a damp KimWipe after each electroporation to remove any bubbles that may have formed. If they are not removed, a mini-charge builds up, which will attract the tissue toward the electrode. The electroporation parameters should be set to ten consecutive 50 msec pulses, at 15 V with a 1 sec pause between pulses.
4. Place the fish on a glass slide or Petri dish and quickly image the fin, making sure to note the dorsal/ventral orientation. This image will be used for regrowth analysis on the following day. Return the fish to the tank.

5. Analysis

1. If the targeted protein that was knocked down in expression is required for proper fin regeneration, a dramatic difference between the dorsal and ventral halves of the fin should be evident 1 day post-electroporation (**Figure 5B**).
2. At 3 dpa, take another picture of the fin of each fish and match up this image with the corresponding 2 dpa image (**Figure 5B**). The natural variation in the fin pigmentation pattern between animals will allow you to correctly identify individual fish.
3. Using NIH Image, trace the 2 dpa and 3 dpa areas of both the dorsal and ventral halves (**Figure 5B**).
4. To determine % area of dorsal versus ventral use the following formula: $(\text{Dorsal}_{3\text{dpa}} - \text{Dorsal}_{2\text{dpa}}) / (\text{Ventral}_{3\text{dpa}} - \text{Ventral}_{2\text{dpa}}) \times 100 = \% \text{ area}$

6. Representative Results

1. We always assay for fin outgrowth at 3 dpa, or 24 hpe (hours post electroporation). At this time, the fluorescein-tagged morpholino should be present in the dorsal half of the fin (**Supplemental Figure 1** and **Figure 6A**). It is normal to see some trailing down to the level of the amputation.
2. There should be no difference between the dorsal and ventral sides in the fin injected and electroporated with the control morpholinos (**Figure 6B**).
3. If the experimental morpholino targets an essential protein for fin outgrowth, a dramatic difference between the dorsal and ventral sides should be observed (**Figure 6C**).
4. If the electrode touched the fin during electroporation, the tissue will appear brown (almost burnt in appearance) and necrotic.

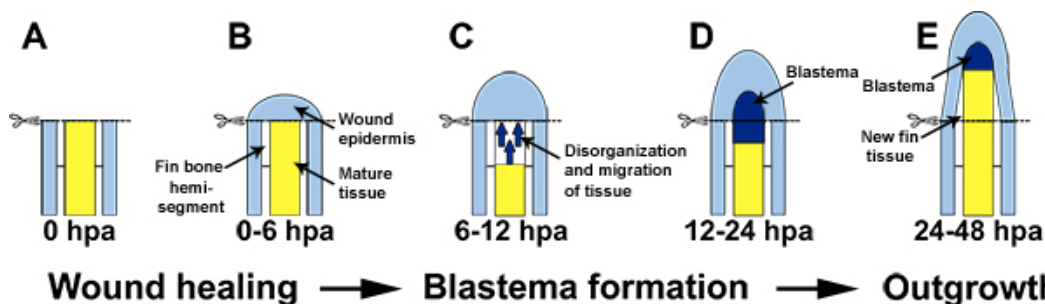


Figure 1. Schematic of the various events that occur during fin regeneration. The underlying times for each event are given in hours post-amputation (hpa) and correspond to a tank temperature of 33 °C.

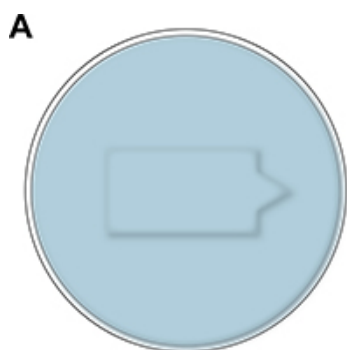


Figure 2. A. Schematic of the injection plate, which is made from agarose and contains a small well to hold the fish during microinjection of the morpholino.

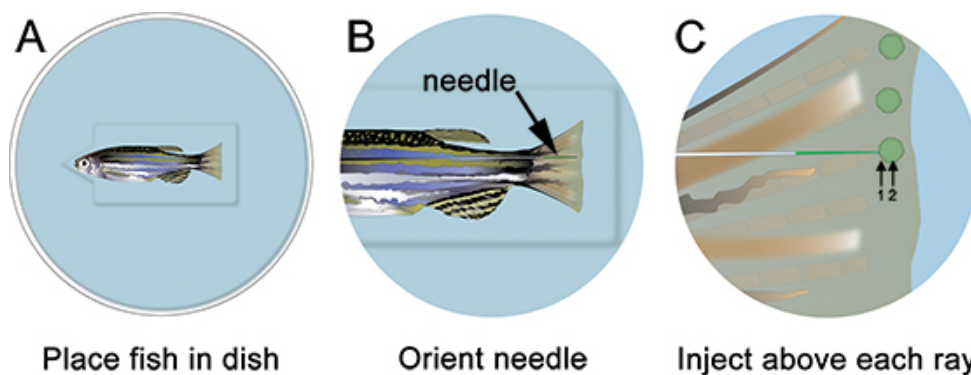


Figure 3. Schematic of morpholino microinjection. **A.** Place the fish in the dish with the head of the fish in the notch cut out of the well, which will help the fish stay stable. **B.** At low magnification, arrange the needle so that it is close to the regenerating tissue of the fin. **C.** At higher magnification, inject the morpholino distal to each bony fin ray (i.e. in each blastema). The needle should enter the tissue just distal to the bony ray (1), and then continue to the location of the blastema (2). Note: the green circles in the schematic are only meant to show the location of the injection. The morpholino can briefly be visualized as a green/yellow "puff" following each injection; however, this does not persist as shown in the schematic.

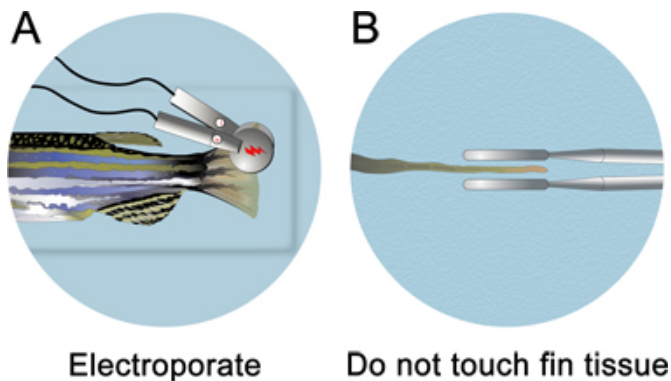


Figure 4. Schematic of fin electroporation. **A.** Following microinjection, place the fish in a Petri dish full of anesthesia and electroporate both the dorsal and ventral halves. **B.** Make sure to not touch the fin tissue. Electrodes should be placed ~ 1 mm from the tissue.

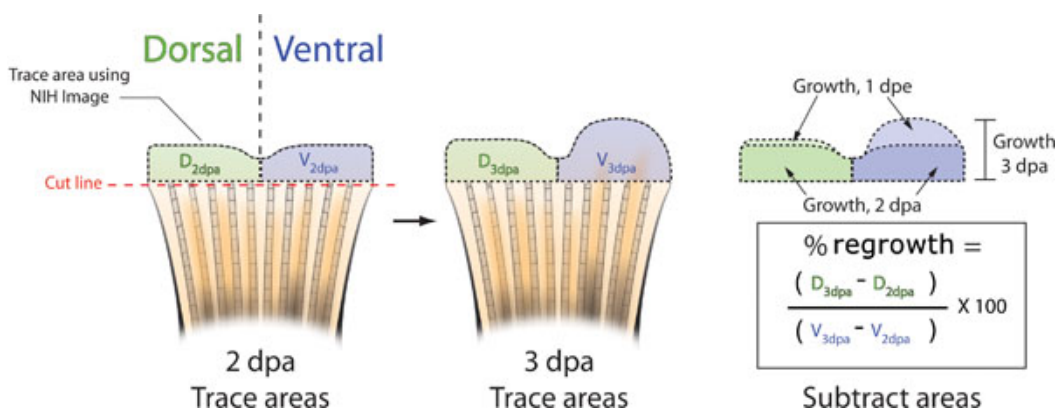
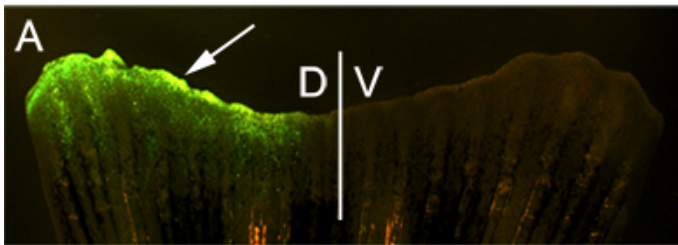
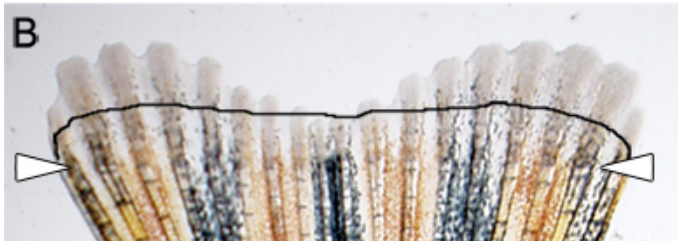


Figure 5. Schematic of the methods used to calculate fin outgrowth inhibition. **A.** Take a picture of the fin of each fish at 2 dpa, either immediately before or after morpholino injection and electroporation. Trace the regenerative tissue of both the dorsal (green) and ventral (blue) halves of the fin using NIH Image, (black dashed lines). **B.** At 3 dpa, take another picture of each fin and again trace the dorsal and ventral areas of regrowth using NIH Image. **C.** Subtract the area of regrowth at 2 dpa from the total regrowth at 3 dpa for both the dorsal and ventral halves. The percent area of dorsal versus ventral re-growth can be calculated using the formula: $((D_{3\text{dpa}} - D_{2\text{dpa}})/(V_{3\text{dpa}} - V_{2\text{dpa}})) \times 100$. Percent inhibition = 100 - Percent Area.

Fluorescein-tagged MO, 24 hpe



Control MO, 24 hpe



Experimental MO, 24 hpe

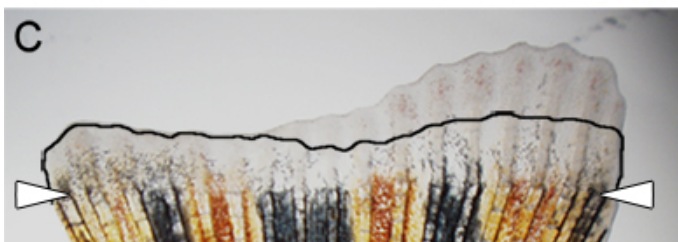


Figure 6. Examples of expected outcomes. **A.** Fluorescent image showing a fluorescein-tagged control morpholino in the dorsal half of the fin, 24 hours post-electroporation (hpe). **B.** Brightfield image of a fin that was injected and electroporated with a control morpholino in the dorsal half. The image shows equal regrowth of both the dorsal and ventral halves of a fin, 24 hpe. **C.** Brightfield image of a fin that was injected and electroporated with an experimental morpholino in the dorsal half. The image shows inhibition of regrowth on the dorsal/injected side. The line shows the amount of regrowth at 2 dpa, immediately prior to morpholino injection and electroporation.

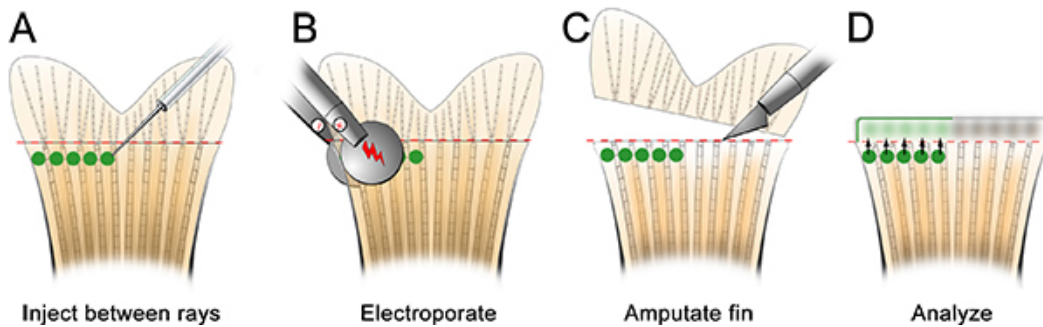


Figure 7. Schematic of an alternate injection and electroporation procedure to target proteins involved in wound healing and blastema formation. **A.** Inject the morpholino between each bony fin ray on the dorsal half of the fin. **B.** Electroporate the morpholino as per normal. **C.** Amputate the fin immediately proximal (~ 1 bony segment) to the injection site. **D.** The fluorescein-tagged morpholino can be observed in wound epithelium and blastema at 24 hpe.

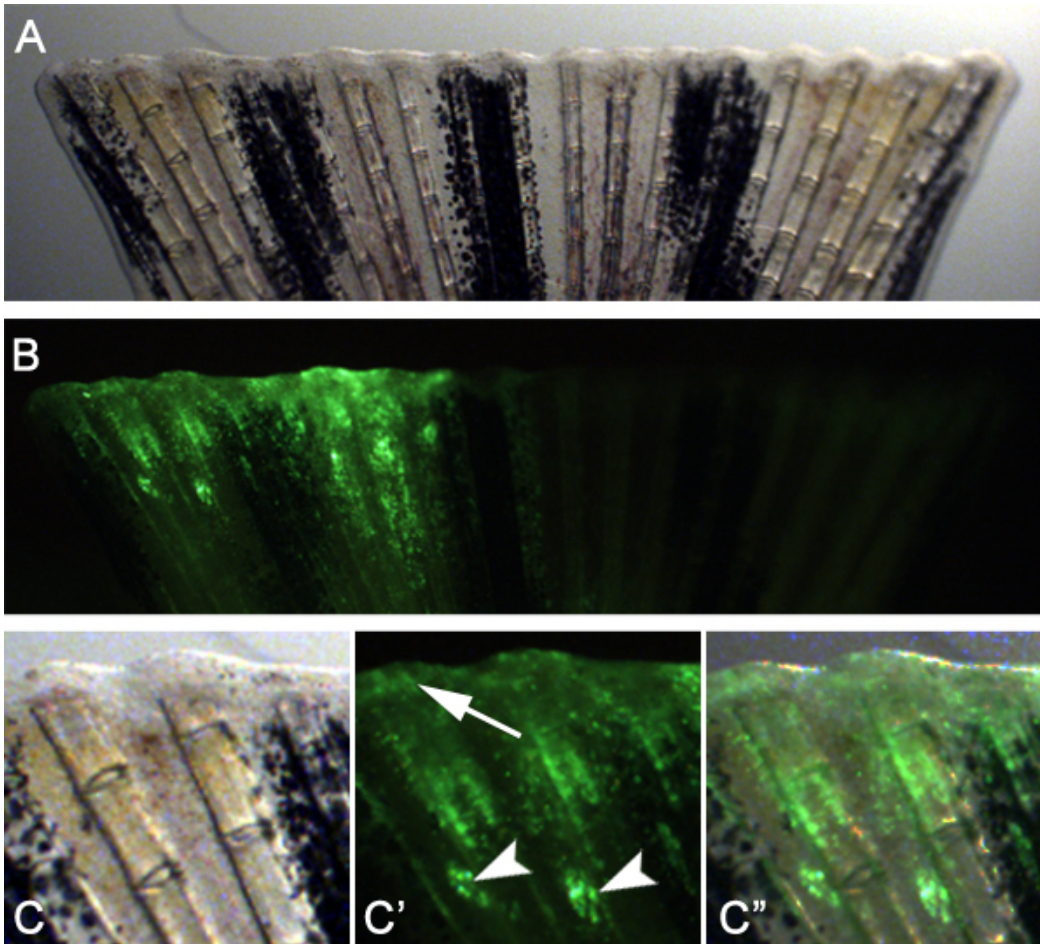


Figure 8. Using the technique to target wound epithelium and blastema formation. **A.** Brightfield image of a fin at 24 hpa that was injected and electroporated with a control morpholino immediately prior to amputation. **B.** Fluorescent image of fin shown in panel A. Note that the injected dorsal half of the fin shows good uptake of the morpholino in the regenerative tissue. **C - C''.** Higher magnification of the dorsal half of the fin shown in panels A and B. The injection sites are often still visible (arrowheads), but many targeted cells have migrated to participate in wound epithelium and blastema formation (arrow).

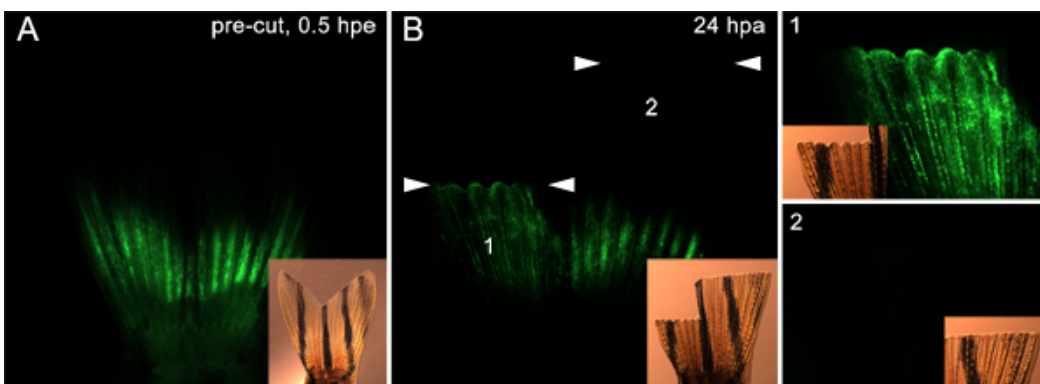


Figure 9. Using the technique to target cells that migrate to form the blastema. **A.** Fluorescent and brightfield inset images showing a fin injected and electroporated with morpholino on both the dorsal and ventral halves. The fin was then amputated at two planes. The dorsal half was cut immediately distal to the injection sites, where as the ventral half was cut 9-10 bony segments distal to the injection sites. **B.** At 24 hpa, the fluorescent and brightfield inset images show that morpholino has migrated to the dorsal regenerate (1), but not the ventral regenerate (2), indicating that only the cell immediately proximal to the cut site participate in blastema formation. The two sets of white arrowheads show the level of each amputation plane. Panels marked 1 and 2 on the far right of the image show a higher magnification view of the dorsal and ventral halves of the fin, respectively.

Supplemental Figure 1. A video of a confocal z-stack of a region corresponding to the location of a blastema in a fin injected and electroporated with a control morpholino. The image was taken at 24 hpe. Since a single fluorescein molecule cannot be visualized, not all of the morpholino

can be visualized or quantified. However, these images give some idea of the varying degrees of uptake that can be visualized in cells, from individual punctate dots, to entire cells full of fluorescent morpholino. On the still image, the orientation is shown. Scale bar: 25 microns.

Discussion

Here, we describe a powerful loss-of-function approach to conditionally knockdown proteins of interest during fin regeneration in adult zebrafish. This technique has been used to study gap junction genes, signaling receptors, transcription factors, and microRNAs during regenerative fin outgrowth^{16, 20-22}.

We anticipate that this technique could also be used to study genes required for wound healing and blastema formation by adapting the technique. For example, we injected and electroporated a control morpholino in the space between the bony fin rays on the dorsal half of the fin prior to amputation (**Figure 7**). We then amputated the fin immediately distal to the injection plane. 24 hpa, we observed that the morpholino-targeted cells had migrated distally to form both the wound epithelium and blastema (**Figure 8**), indicating that cells during these early stages of regeneration can also be targeted.

The technique has a few notable limitations. For example, fluorescence from the fluorescein tag does not persist following fixation and processing for immunohistochemistry, which makes it impossible to correlate a particular cellular phenotype (i.e. cell proliferation) with the amount of morpholino present in a cell. In addition, we have been unable to consistently achieve the electroporation of plasmids into the regeneration tail fin, although a previous group did report the successful electroporation of DNA into fin tissue²³. Finally, we have noted that the morpholino is only effective for ~48 hours post electroporation²¹, which prohibits using this technique in its current form for testing genes involved in the differentiation of new cell types. Additional testing and modification of the procedure may overcome these current limitations.

In addition, it is possible that this technique could be used to test proteins involved in cell migration from the underlying tissue to the blastema. For example, we injected and electroporated a control morpholino into both sides of the fin (as described in **Figure 7**) prior to amputation. We then amputated the dorsal half of the fin immediately distal to the injection plane and we amputated the ventral fin much more distally. At 24 hpa, the morpholino-positive cells on the dorsal side had migrated from the injection site to the overlying wound epithelium and blastema. However, that was not the case on the ventral side (**Figure 9**). This supports the idea that only the cells that underlie the amputation plane participate in the regenerative response. These data also suggest that proteins hypothesized to be required for cell migration could be tested using this technique.

Disclosures

We have nothing to disclose.

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