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TITLE:

Adult Zebrafish Injury Models to Study the Effects of Prednisolone in Regenerating Bone Tissue

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SUMMARY:

Here, we describe 3 adult zebrafish injury models and their combined use with immunosuppressive drug treatment. We provide guidance on imaging of regenerating tissues and on detecting bone mineralization therein.

ABSTRACT:

Zebrafish are able to regenerate various organs, including appendages (fins) after amputation. This involves the regeneration of bone, which regrows within roughly two weeks after injury. Furthermore, zebrafish are able to heal bone rapidly after trepanation of the skull, and repair fractures that can be easily introduced into zebrafish bony fin rays. These injury assays represent feasible experimental paradigms to test the effect of administered drugs on rapidly forming bone. Here, we describe the use of these 3 injury models and their combined use with systemic glucocorticoid treatment, which exerts bone inhibitory and immunosuppressive effects. We provide a workflow on how to prepare for immunosuppressive treatment in adult zebrafish, illustrate how to perform fin amputation, trepanation of calvarial bones, and fin fractures, and describe how the use of glucocorticoids affects both bone forming osteoblasts and cells of the monocyte/macrophage lineage as part of innate immunity in bone tissue.

INTRODUCTION:

Zebrafish represent a powerful animal model to study vertebrate development and disease. This is due to the fact that they are small animals that breed extremely well and that their genome is fully sequenced and amenable to manipulation¹. Other advantages include the option to perform continued live imaging at different stages, including *in vivo* imaging of adult zebrafish², and the ability to perform high throughput drug screens in zebrafish larvae³.

44 Additionally, zebrafish possess a high regenerative capacity in a variety of organs and tissues
45 including bone, and thus serve as a useful system to study skeletal disease and repair^{4,5}.

46
47 Glucocorticoid-induced osteoporosis (GIO) is a disease that results from long term treatment
48 with glucocorticoids, for example in the course of autoimmune disease treatment such as of
49 asthma or rheumatoid arthritis. GIO develops in about 30% of glucocorticoid-treated patients
50 and represents a major health issue⁶; therefore, it is important to investigate the impact of
51 immunosuppression on bone tissue in great detail. In recent years a variety of zebrafish models
52 dealing with the pathogenesis of GIO have been developed. Glucocorticoid-mediated bone loss
53 has been induced in zebrafish larvae, for example, which led to the identification of
54 counteractive compounds increasing bone mass in a drug screen⁷. Furthermore, glucocorticoid-
55 induced bone inhibitory effects have been mimicked in zebrafish scales both *in vitro* and *in*
56 *vivo*^{8,9}. These assays are very convincing approaches, especially when it comes to the
57 identification of novel immunosuppressive and bone anabolic drugs. However, they only partly
58 take into account the endoskeleton and have not been performed in a regenerative context.
59 Thus, they do not allow the investigation of glucocorticoid-mediated effects during rapid modes
60 of adult, regenerative bone formation.

61
62 Here, we present a protocol enabling researchers to study glucocorticoid-mediated effects on
63 adult zebrafish bones undergoing regeneration. Injury models include partial amputation of the
64 zebrafish caudal fin, trepanation of the skull, as well as the creation of fin ray fractures (**Figures**
65 **1A-1C**), and are combined with glucocorticoid exposure *via* incubation (**Figure 1E**). We have
66 recently used a portion of this protocol to describe the consequences of exposure to
67 prednisolone, one of the commonly prescribed corticosteroid drugs, on adult zebrafish
68 regenerating fin and skull bone¹⁰. In zebrafish, prednisolone administration leads to decreased
69 osteoblast proliferation, incomplete osteoblast differentiation and rapid induction of apoptosis
70 in the monocyte/macrophage lineage¹⁰. In this protocol, we also describe how fractures can be
71 introduced into single bony fin ray segments¹¹, as this approach may be useful when studying
72 glucocorticoid-mediated effects on bone occurring during fracture repair. The methods
73 presented here will help to further address underlying mechanisms of glucocorticoid action in
74 rapidly regenerating bone and may also be employed in other settings of systemic drug
75 administration in the context of zebrafish tissue regeneration.

76 77 **PROTOCOL:**

78
79 All methods described here were approved by the Landesdirektion Dresden (Permit numbers:
80 AZ 24D-9168.11-1/2008-1, AZ 24-9168.11-1/2011-52, AZ 24-9168.11-1/2013-5, AZ 24-9168.11-
81 1/2013-14, AZ DD24.1- 5131/354/87).

82 83 **1. Preparation of Materials and Solutions**

84
85 Note: Prednisolone, like other glucocorticoids, leads to immunosuppression. Thus, precaution
86 must be taken to prevent infection in treated animals during the experiment. To this end,

autoclave glass ware and 'fish water' (*i.e.*, the water that is used to rear adult zebrafish) before starting the experiment.

1.1. Calculate the number of zebrafish to be used in the experiment. Be sure to include the individuals that serve as controls.

1.2. Autoclave 600 mL glass beakers that are covered with aluminum foil with autoclave tape. Zebrafish will be treated individually, thus 1 glass beaker is required per zebrafish.

1.3. Calculate the amount of fish water needed for the experiment. 300 mL are required per individual and day. Take into consideration the length of the experiment (*e.g.*, 1 week/7 days).

1.4. Fill glass bottles with fish water, close them and autoclave the bottles with autoclave tape. Ideally, use 2 L bottles, but other bottles like 1 L or 5 L can be used as well.

Note: If experiments are long (*e.g.*, 4 weeks/28 days) and the amount of glass bottles is limited, make sure to prepare bottles for a minimum of several days at a time and repeat the process (1.3 to 1.4) throughout the experiment.

1.5. Prepare a prednisolone stock solution of sterile-filtered 100 mM prednisolone in dimethylsulfoxide (DMSO). Freeze the stock in 1 mL or 2 mL microcentrifuge tube aliquots.

CAUTION: Always wear protective clothing when working with prednisolone (protective gloves, safety goggles, lab coat).

1.6. On the first day of the experiment, prepare incubation solutions with prednisolone and DMSO, respectively. To this end, thaw the required amount of 100 mM prednisolone stock solution and add prednisolone to autoclaved fish water at a final concentration of 50 μ M.

1.6.1. For the control treatments, add the corresponding volume of DMSO to autoclaved fish water. As an example, to produce 2 L prednisolone containing fish water, add 1 mL of 100 mM prednisolone stock to 2 L fish water. In another 2 L bottle of fish water, add 1 mL DMSO, correspondingly.

1.6.2. Work in an appropriate place that is designated to perform drug treatments, ideally in a room at 27 °C.

2. Generation of Injuries in Zebrafish Fins

Note: To injure bone in zebrafish fins, perform resection of the fin (amputation, usually in the caudal fin) or fracture individual bony fin rays (fracture model). To this end anaesthetize adult zebrafish first.

2.1. Amputation

2.1.1. To anaesthetize adult zebrafish, prepare a 100 mm diameter Petri dish containing 0.02% MS-222 (Ethyl-3-aminobenzoate methanesulfonate) in fish water (this water does not need to be autoclaved).

2.1.1.1. Transfer one zebrafish at a time to the MS-222 containing Petri dish by using a fish net and incubate it until it lies on its side without movement and does not respond to touch. Test the latter by touching the anal fin with blunt forceps.

2.1.1.2. Wait until level 4 of anesthesia is reached which is the appropriate level for fin resection¹². At this stage, the rate of opercular movement is decreased, muscle tone is lost and the fish does not move upon touch¹².

2.1.2. Take blunt forceps and transfer the anaesthetized zebrafish carefully to the inversed lid of the 100 mm Petri dish. Place the zebrafish on its lateral side. Make sure the zebrafish always lie in the same orientation, for example on their right body side. With a scalpel or a razor blade, resect 50% of the fin (Figure 1A, 2A).

Note: The speed of zebrafish fin regeneration depends on the amount of resected fin tissue, *i.e.*, the more fin tissue is resected, the faster the fin regenerates¹³. Thus, to minimize variations in fin regeneration make sure to always resect the fin at the same level in all zebrafish.

2.1.3. Transfer the injured zebrafish to an autoclaved glass beaker containing 300 mL of prednisolone or DMSO containing autoclaved fish water. Cover the glass beaker with aluminum foil to avoid escape of the zebrafish.

2.1.4. Repeat steps 2.1.2 to 2.1.3 until the required number of zebrafish for the experiment is reached.

2.2. Fin fracture

2.2.1. Prepare an agarose-coated 100 mm Petri dish by heating a 2% agarose solution in fish water or E3 (embryo media 3, 50 mM sodium chloride, 0.17 mM potassium chloride, 0.33mM calcium chloride, 0.33mM magnesium sulfate). Carefully pour roughly 10-15 mL of the solution into the Petri dish. The bottom of the dish should be completely covered. Let the agarose solidify.

2.2.2. Anaesthetize zebrafish as described in section 2.1.

2.2.3. Take blunt forceps and transfer the anaesthetized zebrafish carefully to the agarose-coated 100 mm Petri dish. Place the zebrafish on its lateral side. Under the dissection microscope, spread the caudal fin completely to be able to identify distinct bony fin rays and fin ray segments. Make sure the zebrafish always lie in the same orientation, for example on their right body side.

2.2.4. With an injection needle (0.3 x 13 mm) introduce a fracture in the center of a bony fin ray segment (**Figure 1B**). To do this, the needle is pushed slightly onto the segment until a crack appears (arrow in **Figure 2B**). While gentle pressure leads to fracture of only one of the two opposing hemirays stronger pressure will lead to fracture of both hemiray segments.

2.2.4.1. Do not introduce too many fractures in fins, since this will greatly impair stability of the fin. As a suggestion, apply fractures only in 3 to 5 fin rays.

2.2.4.2. Always produce fractures in corresponding segments across different fin rays and zebrafish, for example in dorsal fin rays 3, 7 (or 8) and ventral fin ray 3. The proximal-distal level of fracture is ideal at 3 segments proximal to the fin rays' bifurcation point ¹¹.

2.2.5. Transfer the injured zebrafish to an autoclaved glass beaker containing 300 mL of prednisolone or DMSO containing autoclaved fish water. Cover the glass beaker with aluminum foil to avoid escape of the zebrafish.

2.2.6. Repeat steps 2.2.2 to 2.2.5 until the required number of zebrafish for the experiment is reached.

3. Generation of Calvarial Skull Injuries (Trepanation)

Note: The calvariae in zebrafish are homologous to calvarial bones in mammals. Thus, these exoskeletal bones¹⁴ represent a tissue of special interest when studying the pathogenesis of GIO. To injure the skull, trepanation is performed by drilling a hole in the *Os frontale* and/or *Os parietale* (**Figures 1C, 2C**) with the help of a microdrill¹¹.

3.1. Anaesthetize the zebrafish according to section 2.1.

3.2. Hold the anesthetized zebrafish upright with forceps, so that the calvarial bones are well visible under a dissection microscope from above (**Figure 1C**).

3.3. Locate the rotating microdrill on the center of the frontal bone (*Os frontale*) and touch the bone. The produced hole is of the size of the microdrill burr (500 µm, **Figures 2C, 3B**).

Note: Be sure to not move the microdrill laterally while producing the injury. Also stop immediately when the resistance of the bone suddenly drops. Do not drill any further, otherwise the brain will be damaged ¹⁵.

3.4. Transfer the injured zebrafish to a cage filled with fish water until it fully recovers from anesthesia. Watch it carefully.

3.5. Transfer the injured zebrafish to a fish water plus prednisolone or DMSO containing glass beaker.

4. Treatment of Zebrafish During Incubation

Note: During application of prednisolone/DMSO, the drug containing fish water needs to be changed daily, and zebrafish need to be fed regularly.

4.1. Water changes

4.1.1. To exchange the fish water, thaw 100 mM prednisolone stock and prepare the required amount of 50 μ M prednisolone in autoclaved fish water fresh every day of the treatment period. Prepare DMSO containing fish water for the control zebrafish accordingly.

4.1.2. Take a glass beaker containing a single housed zebrafish in its incubation solution and transfer the solution including the zebrafish into an appropriate container, for example a temporal housing cage.

Note: Always use separate interim containers for prednisolone and DMSO treated zebrafish, respectively, to make sure DMSO control zebrafish do not get exposed to prednisolone and vice versa.

4.1.3. Refill the glass beaker with fresh drug containing fish water (300 mL).

4.1.4. Transfer the zebrafish from the interim container to the glass beaker containing fresh incubation solution using a fish net. Put back the foil to avoid escape of the zebrafish.

4.1.5. If the time of the experiment exceeds 2 weeks, exchange the glass beakers.

4.2. Feeding

4.2.1. During short treatments (for up to 2 days) do not feed zebrafish. During longer experiments, feed zebrafish. Take special care to avoid pollution of the incubation solution. Thus, feed with *Artemia ssp.* rather than with flake food, and only on every second day.

Note: *Artemia* are regularly used to feed young zebrafish, and should be available in the zebrafish haltering unit.

4.2.1.1. Roughly 2 h before the fish water is exchanged, feed zebrafish with *Artemia ssp* in their glass beakers. To do so, use a plastic pipette and feed 0.5 to 1 mL of hatched *Artemia* solution per glass beaker.

4.2.2. Wait for 2 h and exchange the incubation solution with fresh drug containing fish water according to section 4.1.

5. Analyses of Samples

Note: After incubation of injured zebrafish in prednisolone and DMSO containing fish water, respectively, either perform bone mineralization/calcification analyses on fixed tissue (5.1 to 5.3) or carry out live imaging of zebrafish under the dissection microscope (5.4)^{10,11,16}. Use live imaging to determine fin regenerate length and to detect differences in reporter gene expression in transgenic zebrafish.

5.1. Fixation of tissues of interest

5.1.1. For fixation of fins and/or skulls prepare 4% paraformaldehyde (PFA) in PBS and freeze at -20 °C for storage. Use freshly prepared 4% PFA in PBS or thaw the required amount before use. Alternatively, 2% PFA in PBS can be used.

CAUTION: PFA is toxic and should be handled carefully under a fume hood. Wear protective clothing.

5.1.2. For fixation of fins prepare a small Petri dish (e.g., 30 mm) with cold 4% PFA in PBS and make sure that the bottom of the dish is fully covered. For fixation of skulls, prepare a little glass jar with lid or a tube with cold 4% PFA in PBS.

CAUTION: Handle PFA containing solutions only under the fume hood as it is toxic. Wear protective clothing.

5.1.3. Harvest the tissue of interest.

5.1.3.1. To harvest regenerating fin tissue or fins with fractures anaesthetize the zebrafish according to section 2.1 or euthanize the zebrafish according to section 5.1.5.

5.1.3.2. Transfer the anaesthetized zebrafish to the lid of a Petri dish. By using a scalpel or razor blade, harvest the fin regenerate. Make sure to include stump tissue in regenerating fins to facilitate handling of the harvested tissue.

5.1.4. Fix fins flat in the PFA contain Petri dish at 4 °C overnight. Flat fixation avoids curling up of the fin tissue during fixation.

5.1.4.1. To fix flat, grab the fin tissue with one fine forceps at the dorsal or ventral edge of the stump and immerse it in the fixation solution. Grab the opposite edge of the stump with a second fine forceps and slightly depress the tissue onto the bottom of the dish.

5.1.4.2. Hold for 10 to 20 s before releasing. The fin tissue should not curl up anymore. If it does, repeat.

5.1.5. Euthanize zebrafish in order to harvest injured skull tissue. Prepare a 100 mm diameter Petri dish containing 0.1% MS-222 in fish water. Transfer one zebrafish at a time to the MS-222

containing Petri dish by using a fishing net. When level 6 of anesthesia (medullary collapse) is reached wait at least 10 min to assure death of the specimen¹².

5.1.6. Using a scalpel cut off the head plus a little trunk tissue from the rest of the body. Immerse the harvested tissue in the prepared 4% PFA solution in the glass jar or plastic tube. Fix for 24 h at 4 °C with gentle rocking.

5.1.7. To store samples long-term after fixation, wash them with PBS (3 x 20 min at RT) and methanol-treat in an increasing methanol series (1x 30%, 1x 50%, 1x 70%, 2x 100% methanol in PBS for 20 min each).

5.1.7.1. Store them in 100% methanol at -20 °C. Otherwise, directly proceed with bone staining or other analyses after washing with PBS.

5.2. Bone staining techniques I (alizarin red staining)

Note: This staining is performed according to van Eeden *et al.* 1996¹⁷ and Walker & Kimmel 2007¹⁸ with modifications.

5.2.1. To perform alizarin red staining on adult fins or skulls, harvest and fix tissue as described in sections 5.1.

5.2.2. If samples were stored in methanol at -20 °C, rehydrate samples by doing an inverse methanol series (70%, 50% and 30% in PBS, each 1x20 min) and wash twice 20 min with PBS. Wash samples for 5 min minimum with deionized water.

5.2.3. To do alizarin red staining in skulls, treat samples with 50 mg/mL trypsin in 30% saturated Na₂B₄O₇ at room temperature overnight. Rock samples during this time. Rinse samples in 30% saturated Na₂B₄O₇ solution. Wash samples with deionized water 2 times for 5 min each. This step is not performed for fin tissue.

5.2.4. Add alizarin red solution (Part B: 0.5% alizarin red S powder, 10% glycerol in deionized water) and rock the samples during incubation at RT. Check for staining after 1, 2, 4 h or overnight.

5.2.5. To clear samples treat them with a decreasing 1% potassium hydroxide:glycerol series (3:1 overnight, 1:1 overnight, 1:3 overnight).

5.2.6. Store the samples in a 1:1 solution of 0.1% potassium hydroxide:80% glycerol and mount them with 80% glycerol for imaging.

Note: For combined Alcian blue/alizarin red staining treat samples with Alcian blue staining solution (Part A: final 0.02% Alcian blue, 50 mM MgCl₂, 70% ethanol) after rehydration (section 5.9) for 2 h at RT. Treat fins with a decreasing ethanol series in 50 mM MgCl₂ (70%, 50%, 30%,

each 1x 15 min) and wash them for a minimum of 1h (3x 20 min) in deionized water. Proceed with alizarin red staining (section 5.2).

5.3. Bone staining techniques II (calcein staining)

Note: To detect calcium deposition in fin fractures and skull injuries, live fish are incubated in calcein containing solution.

5.3.1. Prepare the required amount of 0.2% calcein in deionized water (pH 7.5). Make sure the pH is neutral. Use 1 M NaOH to adjust the pH.

5.3.2. Incubate up to 3 zebrafish simultaneously in 100 mL of calcein solution for 20 min in a glass beaker covered with aluminum foil.

5.3.3. Transfer the zebrafish to a beaker with fish water. Let them swim briefly before transferring them to a new beaker with fish water. Let them swim in this new beaker for 20 min to wash. Take pictures (see sections 5.4).

5.4. Live imaging of zebrafish

5.4.1. Use this approach to acquire images of regenerating bone tissue such as in the fin or skull throughout the experiment without sacrificing the zebrafish. Anaesthetize the zebrafish according to section 2.1.

5.4.2. To acquire images of fin regenerates, transfer the anaesthetized zebrafish onto an agarose-coated Petri dish (see section 2.7 and **Figure 1B**).

5.4.3. To acquire images of regenerating skull bones, place the zebrafish upright in a sponge (**Figure 1D**) or place it in an agarose-coated dish that has been prepared to hold the trunk of the zebrafish.

5.4.4. Acquire images at the desired magnification and resolution using a stereomicroscopic setup.

5.4.5. Return the zebrafish to a container with fresh or drug containing fish water.

REPRESENTATIVE RESULTS:

The protocol presented here has been used repeatedly to induce rapid bone formation in the course of regeneration of the zebrafish fin and skull^{10,11,16}. In combination with the presented method of prednisolone administration, studies on prednisolone's effects during bone regeneration can be pursued. For example, studies on bone formation and mineralization in the regenerate can be performed. Prednisolone, as other glucocorticoids^{19,20}, leads to overall inhibition of fin regeneration, including bone formation, as detected by alizarin red staining on fixed caudal fin tissue (**Figure 3A**). Similarly, prednisolone has a delaying effect on (calvarial)

skull injury closure, which can be illustrated by alizarin red (**Figure 3B**) or *in vivo* calcein staining (not shown). In addition, prednisolone exerts a profound anti-inflammatory effect in both fin and skull tissue, by triggering apoptosis in the monocyte/macrophage lineage. Reduced macrophage numbers can be detected by immunohistochemistry on frozen tissue sections, *e.g.*, by using an anti-mcherry antibody in transgenic *mpeg1:mCherry* zebrafish (**Figure 3C**)^{10,21}. Similarly, the number, distribution, proliferation and apoptosis of other cell types of interest both in the exo- and the endoskeleton (*e.g.*, vertebrae) can be analyzed with the help of immunohistochemistry.

FIGURE LEGENDS:

Figure 1: Procedures carried out in zebrafish. **A.** Resection of the caudal fin (amputation) in anaesthetized zebrafish with the help of a scalpel. The red dashed line indicates the amputation level. **B.** Fin ray fracture in anaesthetized zebrafish carried out with an injection needle under the stereomicroscope. The fish is laying on an agarose-coated Petri dish during the procedure. Agarose-coated Petri dishes are also used to acquire images of zebrafish fins during regeneration or fracture repair. **C.** Trepanation of the calvariae (skull injury) performed in anaesthetized zebrafish with the help of a microdrill under the stereomicroscope. **D.** Image acquisition of regenerating skull bone after injury. The anaesthetized zebrafish is placed upright in a sponge and images are acquired with the help of a stereomicroscope. The Petri dish is filled with fish water containing 0.02% MS-222. **E.** Incubation of zebrafish in prednisolone or DMSO containing fish water.

Figure 2: Microscopic live view of injuries at 0 h post injury (hpi). **A.** Amputated caudal fin. Scale bar 200 μ m. **B.** Fractured fin ray. The fracture is indicated by the red arrow. Scale bar 100 μ m. **C.** Trepanated skull. The injury site is indicated by the white arrowhead. Scale bar 500 μ m.

Figure 3: Representative results of prednisolone treatment in adult zebrafish. **A.** Alizarin red stained fin regenerates at 14 days post amputation (dpa) and days of treatment (dt). Prednisolone treated fin regenerates are shorter (not shown), and a smaller domain of alizarin red positive bone matrix is detected. Scale bar 500 μ m. **B.** Alizarin red stained skulls at 7 days post injury (dpi) and dt. Scale bar 100 μ m. This figure has been modified with permission¹⁰. **C.** Cryosection view of uninjured skull and brain tissue of treated (prednisolone) and untreated (DMSO) Tg (*mpeg1:mCherry*) x Tg (*osterix:nGFP*) transgenic reporter fish, in which macrophages (innate immune cells) are labeled in red²¹ and bone forming osteoblasts are labeled in green²². The number of macrophages significantly drops in prednisolone treated zebrafish. Immunohistochemistry was performed as described in Geurtzen *et al.*¹⁰. Nuclei are labeled in blue (DAPI). Scale bar 20 μ m. BF = bright field, epid = epidermis, bn = bone. This figure has been modified with permission¹⁰.

DISCUSSION:

Zebrafish have proven useful in skeletal research in many regards. Selected mutants mimic aspects of human disease such as osteogenesis imperfecta or osteoarthritis²³⁻²⁷, and larvae as well as scales are being used to identify bone anabolic compounds in small molecule screens^{7,28,29}. Treatments of zebrafish with drugs that are applied in clinical practice are

furthermore ideally suited to study putative adverse effects, for example in bone tissue. In this context, the presence of rapidly regenerating bone is advantageous to investigate the underlying mechanisms of medication-induced bone deficiencies. Here, we present a protocol in which drug treatment with the glucocorticoid prednisolone, a commonly used anti-inflammatory drug with adverse effects in bone tissue, is combined with adult bone regeneration regimes. This protocol has successfully been used to induce immunosuppressive and bone inhibitory effects in regenerative tissues of zebrafish, and can also be adapted for studies on the impact of prednisolone and other drugs in adult such as the spine. The following details should be taken into account when immunosuppressive drug treatments are performed in zebrafish undergoing fin or skull regeneration.

Reproducibility of injury assays

During fin regeneration, regeneration speed (*i.e.*, regrowth of fin tissue, including bone, per time unit) depends very much on the amount of fin tissue that is being resected¹³. In order to avoid unwanted variability of fin regeneration, make sure to always resect equivalent amounts of fin tissue in all specimens.

Execution of bony fin ray fractures is carried out by slightly pushing an injection needle onto the center of a chosen bony fin ray segment. As bony fin rays consist of 2 opposing hemirays, the amount of pressure used determines whether only one or both hemirays are fractured. We prefer to apply low pressure to fracture only one hemiray, because fracture of both hemirays tends to destabilize the fin ray, which as a result may detach during the following day(s). Calvarial injuries of the skull by microdrilling should be performed cautiously. If damage is done to the brain (*i.e.*, the cerebellum) postural and locomotory deficits will become apparent by erratic swimming of the specimen¹⁵. Zebrafish with cerebellar injury may show adverse reactions to drug treatment and should not be used to study bone regeneration.

Considerations regarding drug treatments

To produce a consistent immunosuppressive and anti-regenerative effect in adult zebrafish we employ a dose of 50 μ M prednisolone in fish water. We identified this dose during initial dose-response experiments in larval and adult zebrafish. Thus, dose-response experiments should be carried out to identify the required dose of other immunosuppressive agents that might be applied. For adults, we recommend combining these initial experiments with the fin regeneration regime, as fin regenerate length is a highly sensitive and easy-to-detect readout for tissue alterations.

Immunosuppressive treatment predisposes treated specimens to microbial infections. Therefore, single housing in autoclaved beakers containing autoclaved fish water is important. Although these conditions are more cumbersome to carry out and are not sterile, they help to minimize infection in treated zebrafish. We did not pretreat ('sterilize') *Artemia* eggs. However, this might be an additional measure to prevent infection in zebrafish, if necessary. Furthermore, antiseptic substances such as methylene blue (1%) can be added to fish water before use.

Experiments with prednisolone, both short- and long-term, require daily changes of fish water. We have treated adult zebrafish for up to 8 weeks. Treatment of a large number of individuals for such a long time can lead to a certain experimental 'burden', and should be planned carefully. It is pivotal to always have the required amounts of autoclaved fish water and glass ware ready. Although this has not been tested in zebrafish (to our knowledge), implantation of slow release pellets for drugs of interest might represent a valuable alternative for long term drug exposure in zebrafish.

Analyses in transgenic zebrafish

Here, we present 2 methods to stain for mineralization/calcification of bone tissues by alizarin red and calcein staining. Additionally, we show how to image regenerating fin and skull tissue *in vivo* with the help of a stereomicroscope. The latter technique is very useful, if transgenic zebrafish reporting the number or activity of selected cells, such as osteoblasts or immune cells, are being imaged. For example, before sacrificing injured and prednisolone-treated zebrafish to perform alizarin red staining, fin regenerates or trepanated skulls undergoing repair can be photographed to look for the presence, number and activity of bone forming osteoblasts in the transgenic reporter line Tg (*osterix:nGFP*)²². Likewise, epidermal wound closure, which occurs within a day, can be visualized in transgenic fish, which express a fluorophore in epidermal tissue. Also, accumulation of immune cells at the site of injury (or their absence in prednisolone treated individuals) can be monitored easily with a stereomicroscope equipped with the required light source and filters.

In sum, the protocol presented here can be used to study effects of immunosuppressive agents and other drugs after systemic administration in zebrafish that are undergoing bone regeneration either in the fin or skull. This will be useful to delineate the pathogenesis of GIO and to investigate the mechanisms underlying successful bone regeneration.

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DISCLOSURES:

The authors have nothing to disclose.

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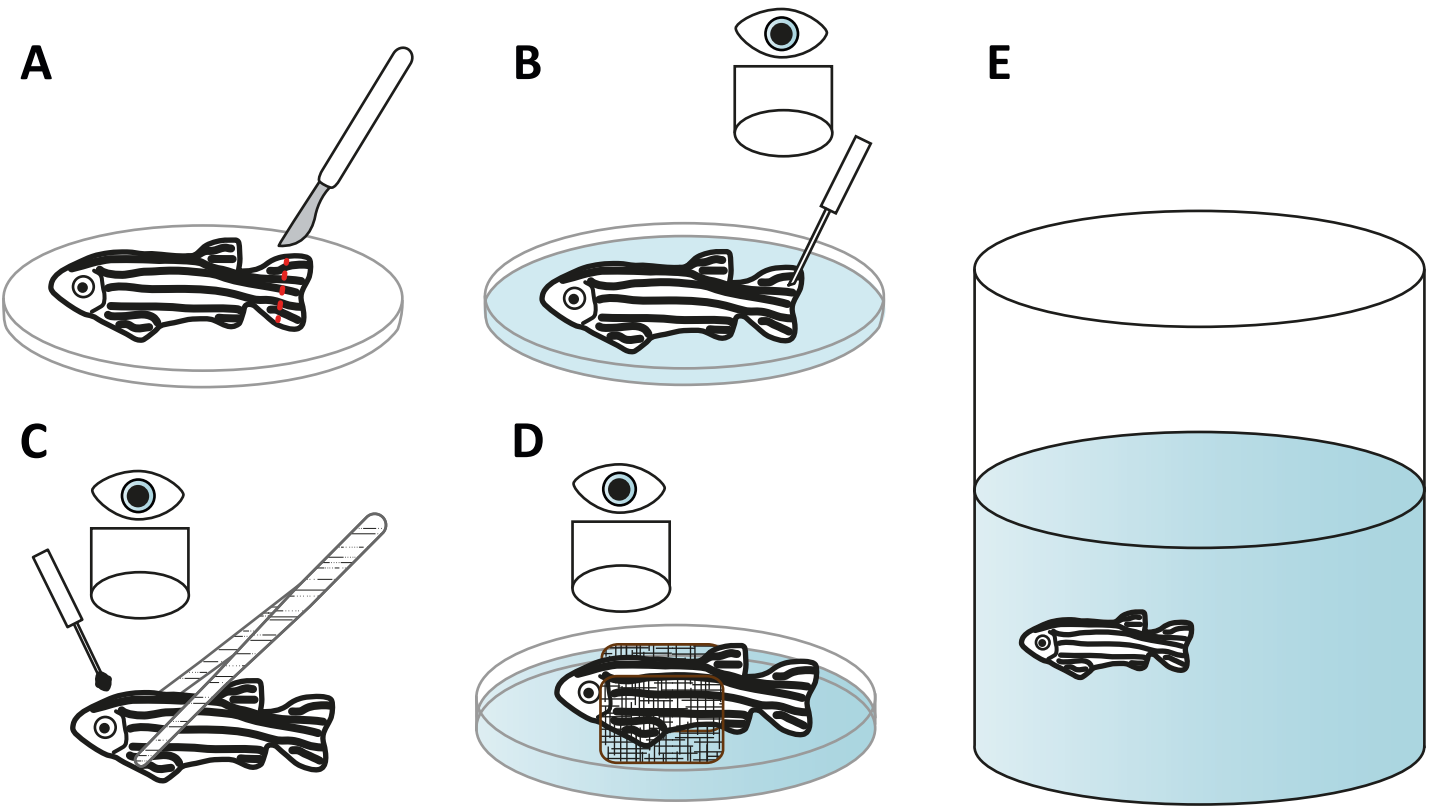
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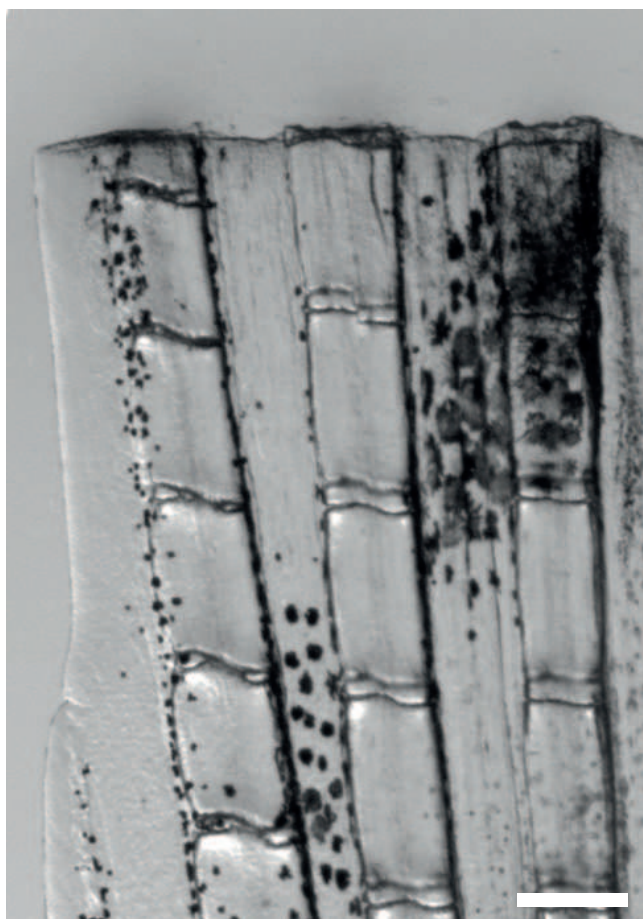
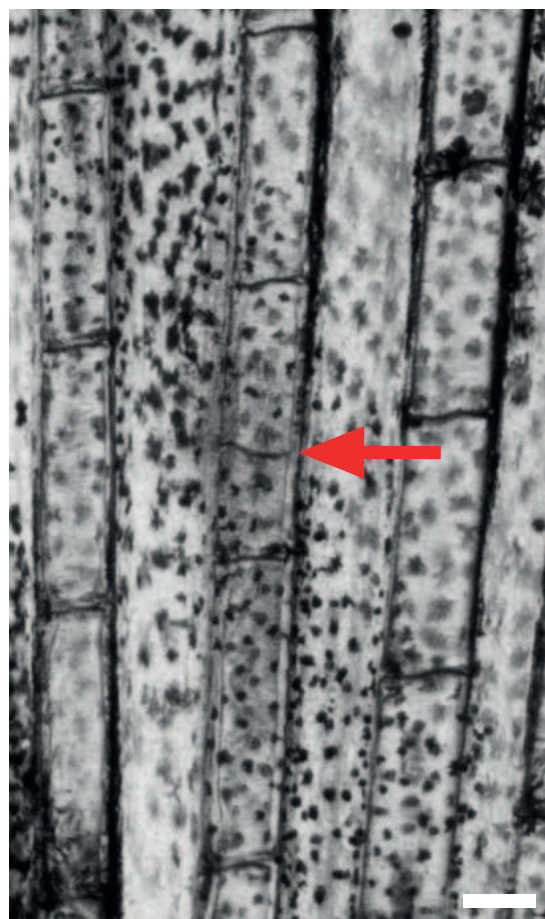
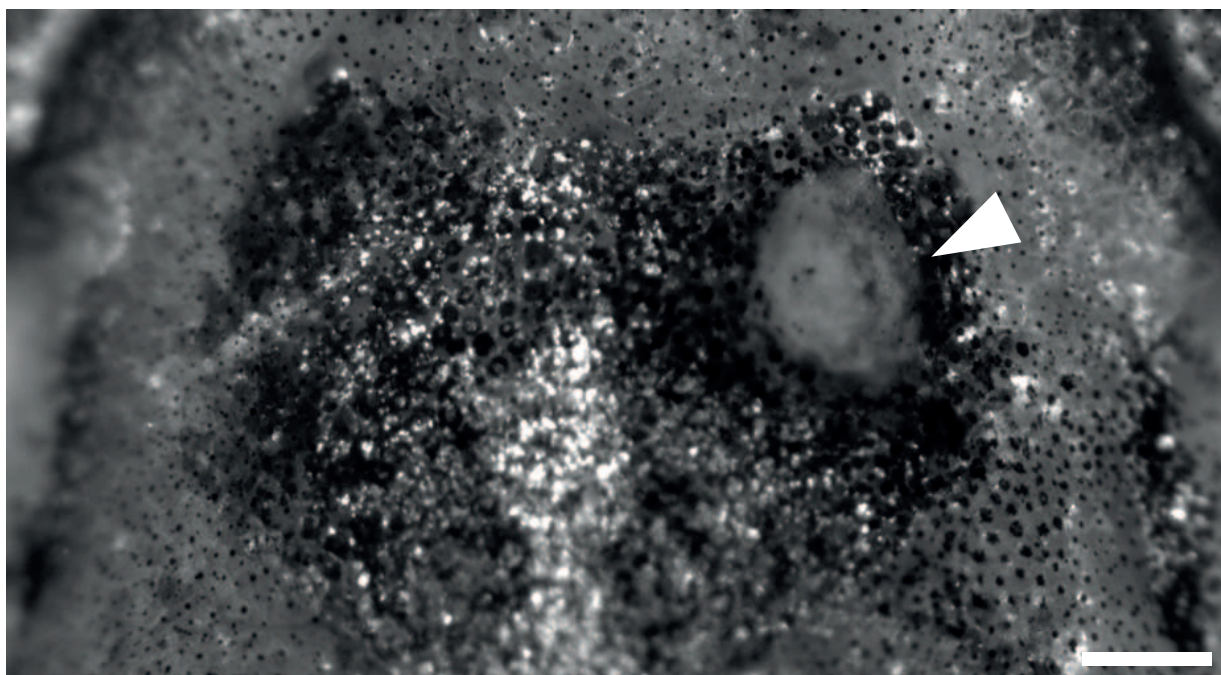
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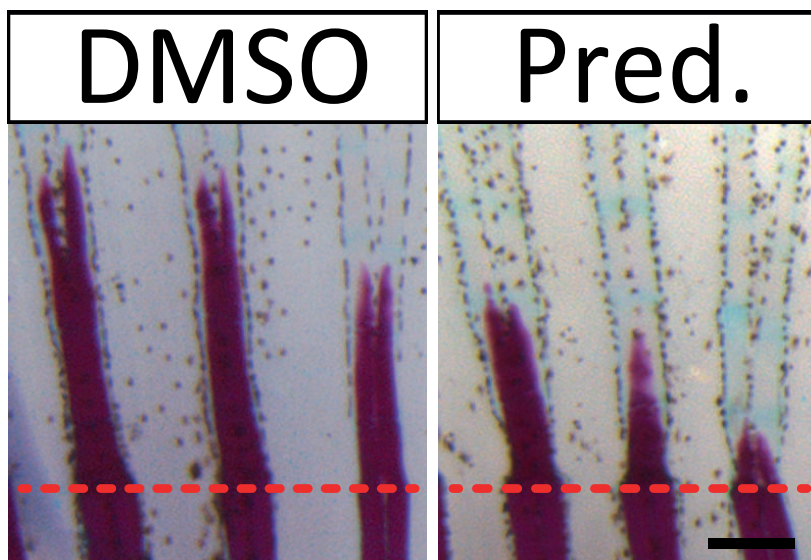
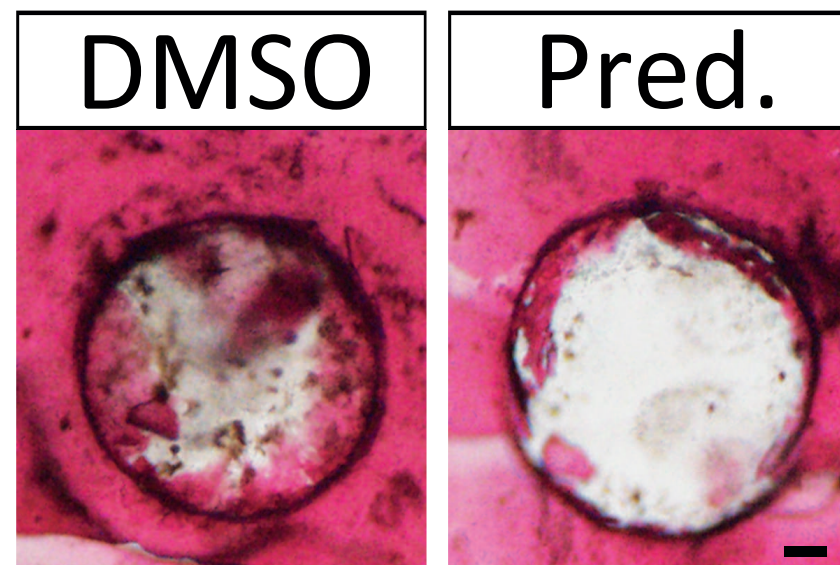
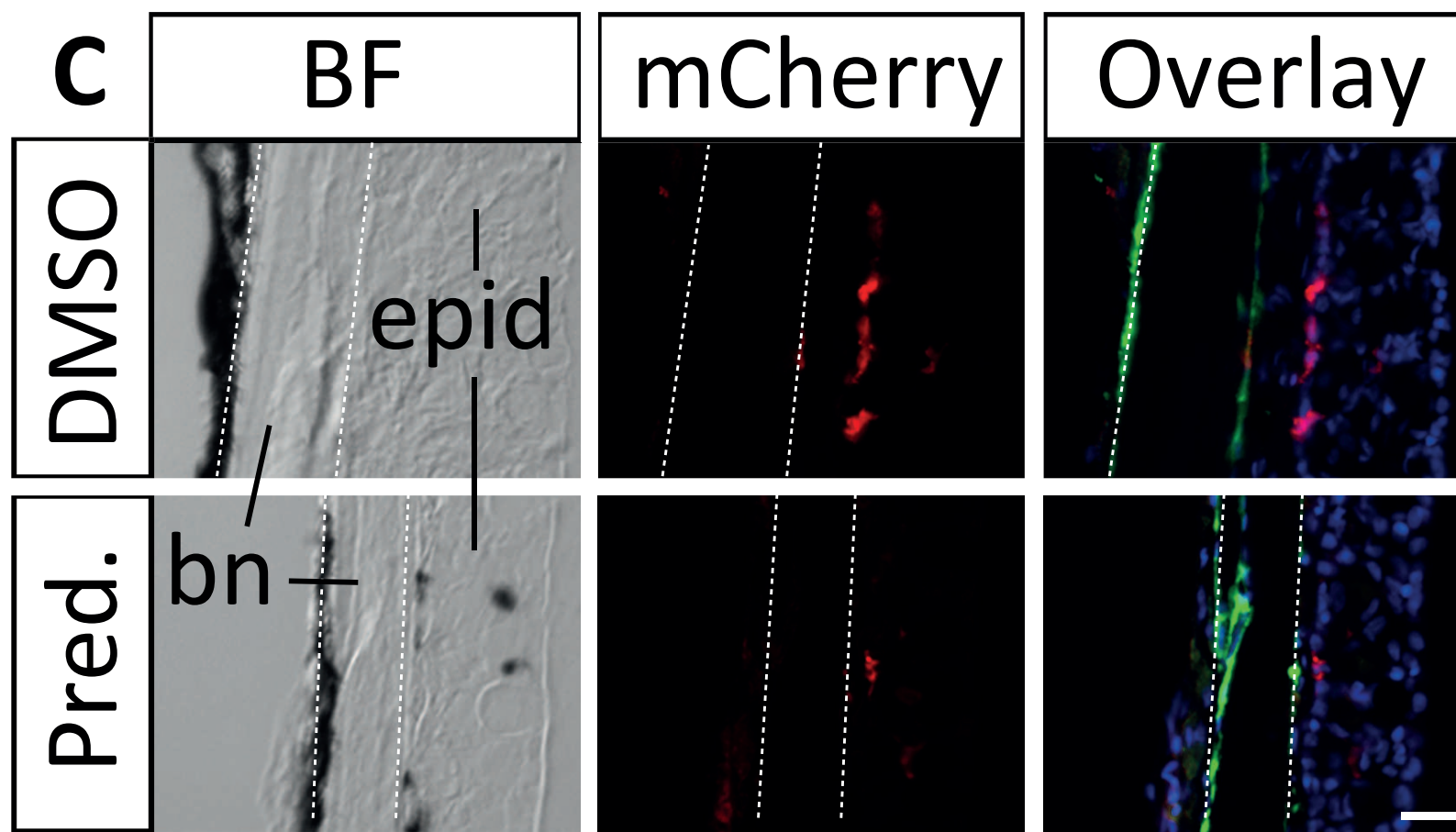
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Prednisolone	Sigma-Aldrich	P6004	
Dimethylsulfoxid (DMSO)	Sigma-Aldrich	D8418	
Ethyl-3-aminobenzoate methanesulfonate (MS-222)	Sigma-Aldrich	A5040	
Blunt forceps	Aesculap	BD027R	
Fine forceps	Dumont	91150-20	
Scalpel	Braun	5518059	
Agarose	Biozym	840004	
Injection needle (0.3x13 mm)	BD Beckton Dickinson	30400	
Micro drill	Cell Point Scientific	67-1000	distributed e.g. by Harvard Apparatus
Steel burrs (0.5 µm diameter)	Fine Science tools	19007-05	
<i>Artemia ssp.</i>	Sanders	425GR	
Pasteur pipette (plastic, Pastette)	Alpha Labs	LW4111	
Paraformaldehyde	Sigma-Aldrich	158127	
Alizarin red S powder	Sigma-Aldrich	A5533	
Alcian blue 8 GX	Sigma-Aldrich	A5268	
Calcein	Sigma-Aldrich	C0875	
Trypsin	Sigma-Aldrich	T7409	
Stereomicroscope	Leica	MZ16 FA	with QIMAGING RETIGA-SRV camera

Stereomicroscope	Olympus	MVX10	with Olympus DP71 or DP80 camera
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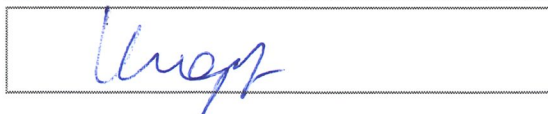
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Response to editorial comments and reviewers' comments

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
 - We have proofread the manuscript thoroughly and also asked an English native speaker to correct grammar and spelling issues.
2. Note that, in the signed Author License Agreement (ALA), two boxes are checked in item 2. Please check one box only, sign and upload the revised ALA to your Editorial Manager account.
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 - We have done accordingly.
4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.
 - We thank the editors for this comment, but are convinced that we have stated the purpose of the method clearly in the introduction. In L63/64 we write: " Here, we present a protocol enabling researchers to investigate glucocorticoid-mediated effects in adult zebrafish bones undergoing regeneration. " In L73-76 we furthermore add: " The methods presented here will help to further address underlying mechanisms of glucocorticoid action in rapidly regenerating bone and may also be employed in other settings of systemic drug administration in the context of zebrafish tissue regeneration."
5. Please use SI abbreviations for all units: L, mL, μ L, h, min, s, etc.
 - We have corrected the abbreviations to SI standards wherever necessary.
6. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.
 - We have included spaces accordingly.
7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
 - We have replaced personal pronouns in the protocol section and restricted their limited use to the introduction and discussion part.
8. 2.1: Please specify what level 4 of anesthesia is.
 - We have added the following sentence in section 2.1 (L131-133): "At this stage, the rate of opercular movement is decreased, muscle tone is lost and the fish does not move upon touch ¹²."

9. Lines 226-230: Please write the text in the imperative tense and include it in step 4.6.
- In the edited version of the manuscript (Lines 233 to 236) we have included the text in step 4.6 and have written it in the imperative tense:

" Feeding

4.6 During short treatments (for example 2 days) do not feed zebrafish. During longer experiments feed zebrafish. Take special care to avoid pollution of the incubation solution. Thus, feed with *Artemia ssp.* rather than with flake food, and only on every second day. *Artemia* are regularly used to feed young zebrafish, and should be available in your zebrafish haltering unit."

10. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion. See lines 240-245, 329-332.

- We thank the editors for this suggestion. We have changed these paragraphs:

The section in lines 329-332 (original version of the manuscript) we have now included in protocol step 5.17 in the imperative tense (L339-341):

"Live imaging of zebrafish

5.17 Use this approach to acquire images of regenerating bone tissue such as in the fin or skull throughout the experiment without sacrificing the zebrafish. Anaesthetize the zebrafish according to section 2.1."

The paragraph in lines 240-245 (original version of the manuscript) is necessary to inform the reader on the 2 following types of protocols that are possible to do. We have written this paragraph in the imperative tense (L244-249):

"5. Analyses of samples

After incubation of injured zebrafish in prednisolone and DMSO containing fish water, respectively, either perform bone mineralization/calcification analyses on fixed tissue (5.1 to 5.16) or carry out live imaging of zebrafish under the dissection microscope (5.17 to 5.21) ^{10, 11, 15}. Use live imaging to determine fin regenerate length and to detect differences in reporter gene expression in transgenic zebrafish."

Similarly, we have instructed the reader to either perform fin resection (amputation) or fin fracture to injure bone in the fin (L120-123):

"2. Generation of injuries in zebrafish fins

To injure bone in zebrafish fins, perform resection of the fin (amputation, usually in the caudal fin) or fracture individual bony fin rays (fracture model). To this end anaesthetize adult zebrafish first."

11. 5.10: Please explain what RT O/N and dH2O mean.

- We have replaced the term RT O/N, which is an abbreviation, with the full phrase 'room temperature over night' in the revised manuscript, and the abbreviation O/N with 'over night'. We have also replaced the term dH2O by 'deionized water'.

12. 5.14: Please specify how to adjust pH if it is not neutral.

- We have added the sentence "Use 1 M NaOH to adjust the pH." to section 5.14 (L328-329).

13. 5.19: Should be Figure 1D instead of Figure 1C.

- We have corrected the mistake.

14. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

- We have reduced the filmable content to 2.75 pages.

15. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing euthanasia.

- We have done accordingly.

16. Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol.

- We have revised the representative results accordingly, i.e. we made clear which types of analyses have been performed in/for the respective figure. We have also suggested further alternative analyses, e.g. regarding cell proliferation. The paragraph now reads the following (L355-370):

"REPRESENTATIVE RESULTS:

The protocol presented here has been used repeatedly to induce rapid bone formation in the course of regeneration of the zebrafish fin and skull ^{10, 11, 15}. In combination with the presented method of prednisolone administration, studies on prednisolone's effects during bone regeneration can be pursued. For example, studies on bone formation and mineralization in the regenerate can be performed. Prednisolone, as other glucocorticoids ^{18, 19}, leads to overall inhibition of fin regeneration, including bone formation, as detected by alizarin red staining on fixed caudal fin tissue (**Figure 3A**). Similarly, prednisolone has a delaying effect on (calvarial) skull injury closure, which can be illustrated by alizarin red (**Figure 3B**) or in vivo calcein staining (not shown). In addition, prednisolone exerts a profound anti-inflammatory effect in both fin and skull tissue, by triggering apoptosis in the monocyte/macrophage lineage. Reduced macrophage numbers can be detected by immunohistochemistry on frozen tissue sections, e.g. by using an anti-mcherry-antibody in transgenic *mpeg1:mCherry* zebrafish (**Figure 3C**) ^{10, 20}. Similarly, the number, distribution, proliferation and apoptosis of other cell types of interest both in the exo- and the endoskeleton (e.g. vertebrae) can be analyzed with the help of immunohistochemistry."

17. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

- Critical steps within the protocol are discussed in the paragraphs "Reproducibility of injury assays" and "Considerations regarding drug treatments" of the discussion. The critical steps include the resection of fins at always the same level (L425-428; "During fin regeneration, regeneration speed (i.e. regrowth of fin tissue, including bone, per time unit) depends very much on the amount of fin tissue that is being resected ¹³. In order to avoid unwanted variability of fin regeneration, make sure to always resect equivalent amounts of fin tissue in all specimens."), the fact that the brain can be damaged during trepanation, which should be avoided (L434-437; "Calvarial injuries of the skull by microdrilling should be performed cautiously. If damage is done to the brain (i.e. the cerebellum) postural and locomotory deficits will become apparent by erratic swimming of the specimen ¹⁵. Zebrafish with cerebellar injury may show adverse reactions to drug treatment and should not be used to study bone regeneration."), and vulnerability of immunosuppressed zebrafish (L447-453; "Immunosuppressive treatment predisposes treated specimens to microbial infections. Therefore, single housing in autoclaved beakers containing autoclaved fish water is important. Although these conditions are more cumbersome to carry out and are not sterile, they help to minimize infection in treated zebrafish. We did not pretreat ('sterilize') Artemia eggs. However, this might be an additional measure to prevent infection in zebrafish, if necessary. Furthermore, antiseptic substances such as methylene blue (1 %) can be added to fish water before use. "

b) Any modifications and troubleshooting of the technique

- We discuss troubleshooting of immunosuppressive treatment in the paragraph "Considerations regarding drug treatments" of the discussion (L447-453, see above). Increased mortality due to infections was the main occasion on which we needed to troubleshoot.

c) Any limitations of the technique

- We mention in the discussion that long term treatment by immersion in individual beakers is cumbersome and that alternative methods, such as prednisolone pellet implantation might be superior to our technique (L454-460; "Experiments with prednisolone, both short- and long-term, require daily changes of fish water. We have treated adult zebrafish for up to 8 weeks. Treatment of a large number of individuals for such a long time can lead to a certain experimental 'burden', and should be planned carefully. It is pivotal to always have the required amounts of autoclaved fish water and glass ware ready. Although this has not been tested in zebrafish (to our knowledge), implantation of slow release pellets for drugs of interest might represent a valuable alternative for long term drug exposure in zebrafish.")

d) The significance with respect to existing methods

- In the new version of the manuscript we have included a phrase in the discussion (L417-420) emphasizing the fact that our protocol can be used to study adult endoskeletal bones: "This protocol has successfully been used to induce immunosuppressive and bone inhibitory effects in regenerative tissues of zebrafish, and can also be adapted for studies on the impact of prednisolone and other drugs in adult endoskeletal bones such as the spine." We have put this into context in the introduction part, stating (L54-62):

"Glucocorticoid-mediated bone loss has been induced in zebrafish larvae, for example, which led to the identification of counteractive compounds increasing bone mass in a drug screen ⁷. Furthermore, glucocorticoid-induced bone inhibitory effects have been mimicked in zebrafish scales both in vitro and in vivo ^{8,9}. These assays are very convincing approaches, especially when it comes to the identification of novel immunosuppressive and bone anabolic drugs. However, they only partly take into account the endoskeleton and do not work in a regenerative context. Thus, they do not allow the investigation of glucocorticoid-mediated effects during rapid modes of adult, regenerative bone formation." We very much value other existing methods to study the effects of drugs on the zebrafish skeleton, and would not like to emphasize the significance of our method beyond what we have written.

e) Any future applications of the technique

- We mention the fact that our protocol can be used to study other anti-inflammatory drugs as well as other drugs of interest on 3 different occasions. These are:
 - i. at the end of the introduction (L73-76): "The methods presented here will help to further address underlying mechanisms of glucocorticoid action in rapidly regenerating bone and may also be employed in other settings of systemic drug administration in the context of zebrafish tissue regeneration.",
 - ii. in the section 'Considerations regarding drug treatments' in the discussion (L441-444): "Thus, dose-response experiments should be carried out to identify the required dose of other immunosuppressive agents that might be applied.", and
 - iii. at the very end of the discussion (L476-479): "In sum, the protocol presented here can be used to study effects of immunosuppressive agents and other drugs after systemic administration in zebrafish that are undergoing bone regeneration either in the fin or skull. This will be useful to delineate the pathogenesis of GIO and to investigate the mechanisms underlying successful bone regeneration."

18. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

- We have changed the formatting style of the bibliography to Jove.ens style and hope that this style fulfills the requirements.

Reviewers' comments:

Reviewer #1:

JoVE manuscript review JoVE58429

Title: Adult zebrafish injury models to study the effects of prednisolone in regenerating bone tissue

Authors: Karina Geurtzen, Franziska Knopf

Zebrafish is able to regenerate various organs, including tissues of the appendages (fins) after amputation. Regeneration of appendages and skull accompanies the regeneration of bones, and thus this type of tissue regeneration is a good model for investigating the underlying molecular and cellular mechanism. In this article, the authors describe the use of these 3 injury models, fin amputation, fin ray bone fracture and calvarial skull injury, using adult zebrafish and their combined application with systemic chemical treatment to dissect bone inhibitory and immunosuppressive effects.

Overall, they described the respective injury models and their step-by-step protocols in sufficient detail, so the article is useful for people who would like to follow and/or newly start the similar studies. I do not have any special comments on the manuscript. However, if they could provide videos that show the actual procedures for making respective injuries, it would be very helpful for people who are not familiar to these models.

Response:

- We thank the reviewer for his/her positive response. Of course, we are going to demonstrate the injury procedures in the video version of the article.

Reviewer #2:

Manuscript Summary:

This manuscript is based on two articles previously published by the authors, where damages of the zebrafish skeleton are performed to examine intramembraneous bone regeneration under normal conditions (Development 2014) or upon immune system suppression (JBM 2017).

Here, the authors present a series of detailed protocols allowing three types of bone damages to be performed (fin amputation, trepanation of calvarial bones, and fin fractures) and the subsequent examination of bone regeneration using in vivo staining techniques (including transgenic lines) and histological procedure.

They further show that their protocol can be used to study the effect of drugs inhibiting the function of the immune system, such as prednisolone.

Major Concerns:

I have no major concerns regarding the described protocol.

Response:

- We thank the reviewer for his/her positive evaluation and valuable comments. We have addressed each of the comments below.

Minor Concerns:

While the text reads fluidly, there are many grammatical mistakes and unusual phrasing

that should be corrected. I recommend the text to be edited by a native English speaker. Some examples: L34 "In here"; L45 "genetically highly amenable", L61 "do not perform" etc...

- We have corrected the mistakes and unusual phrasing to our best knowledge with the help of a native English speaker and a person with a long residence time in the UK. We hope that the text now reads well.

L148: Replace "by heating 2% agarose" with "by heating a 2% agarose solution prepared in fish water or E3".

- We thank the reviewer for pointing out this mistake to us. We have replaced the phrase accordingly (L152).

L179: calvaria is classified as exoskeleton. Please correct and cite: Zoological Lett. 2015 Jan 13;1:2. doi: 10.1186/s40851-014-0007-7. Hirasawa & Kuratani.

- We apologize for the confusion of terms. We have corrected the mistake in protocol section 3 and cite Hirasawa & Kuratani 2015 Zoological Letters (L186).

L277 Perhaps "body" is better suited than "corpse"?

- We have replaced the word "corpse" by the word "body" in section 5.6 of the protocol (L283).

L379: Replace "Cryosection view of uninjured skull and brain tissue of treated" by "Cryosection view of uninjured skull and brain tissue of treated (Pred.) and untreated (DMSO)..."

- We thank the reviewer for this suggestion and have changed the sentence accordingly (L397-398).

Fig3: indicate that BF means "bright field" in the legend. Indicate the position of the bone and brain tissues on the bright field image.

- We have changed the legend accordingly (L402). We have also changed Figure 3C and indicated the position of epidermis (epid) and bone (bn) in the bright field image. The brain is not visible on the images since it usually detaches from the bone during fixation and cryosectioning.

- Again, we would like to thank the reviewers and editors for their positive and constructive feedback.



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Title: Immune Suppressive and Bone Inhibitory Effects of Prednisolone in Growing and Regenerating Zebrafish Tissues

Author: Karina Geurtzen, Aude Vernet, Andrew Freidin, et al

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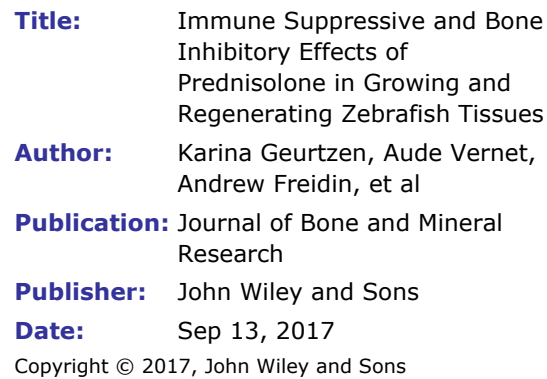
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