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Protocol for Rapid Assessment of the Efficacy of Novel Wnt Inhibitors Using Zebrafish Models

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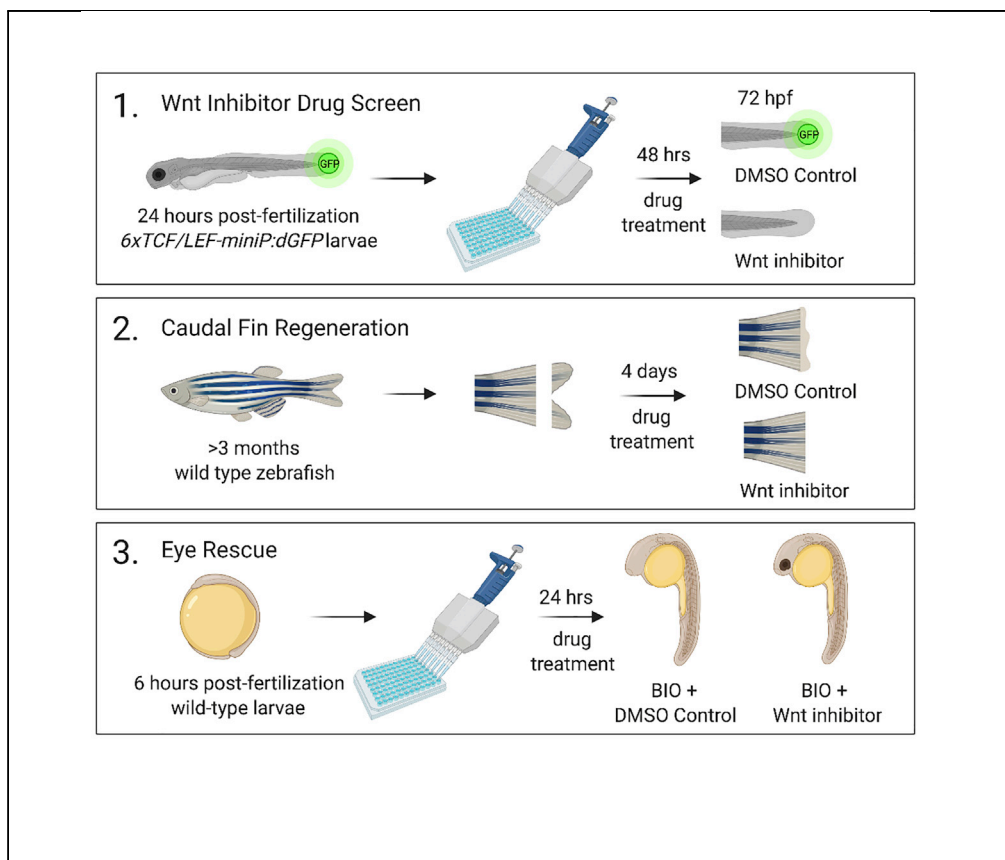
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Protocol

Protocol for rapid assessment of the efficacy of novel Wnt inhibitors using zebrafish models



Dysregulation of Wnt signaling is a hallmark of many cancers, and the development of effective, non-toxic small-molecule Wnt inhibitors is desirable. Off-target toxicities of new compounds are typically tested in mouse models, which is both costly and time consuming. Here, we present a rapid and inexpensive protocol to determine the *in vivo* toxicity and efficacy of novel Wnt inhibitors in zebrafish using a combination of a fluorescence reporter assay as well as eye rescue and fin regeneration assays. These experiments are completed within 1 week to rapidly narrow drug candidates before moving to more expensive pre-clinical testing.

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Highlights

Step-by-step instructions to perform *in vivo* screening of Wnt inhibitors in zebrafish

An *in vivo* fluorescence reporter assay as a readout of Wnt signaling activity

Inhibitor efficacy validated by rescuing Wnt signaling overactivation-mediated phenotypes

Inhibitor efficacy validated by disrupting Wnt-driven zebrafish caudal fin regeneration

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Protocol

Protocol for rapid assessment of the efficacy of novel Wnt inhibitors using zebrafish models

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SUMMARY

Dysregulation of Wnt signaling is a hallmark of many cancers, and the development of effective, non-toxic small-molecule Wnt inhibitors is desirable. Off-target toxicities of new compounds are typically tested in mouse models, which is both costly and time consuming. Here, we present a rapid and inexpensive protocol to determine the *in vivo* toxicity and efficacy of novel Wnt inhibitors in zebrafish using a combination of a fluorescence reporter assay as well as eye rescue and fin regeneration assays. These experiments are completed within 1 week to rapidly narrow drug candidates before moving to more expensive pre-clinical testing.

For complete details on the use and execution of this protocol, please refer to Zhang et al. (2020).

BEFORE YOU BEGIN

Note: *6xTCF/LEF-miniP:dGFP* zebrafish are a transgenic reporter zebrafish line that contains six repeats of the TCF/LEF DNA binding site upstream of a minimal promoter, derived from the PGL4 vector, and destabilized GFP (dGFP) sequence. In cells with activated Wnt signaling, TCF/LEF transcription factors bind to the DNA binding site, leading to transcription of dGFP and fluorescence in those cells (Shimizu et al, 2012). The zebrafish line is maintained by in-crossing, and screening for GFP fluorescence in each generation is required.

Note: The syngeneic CG1 zebrafish line was used for the eye rescue experiment, however, any strain can be used. A pigmented strain is ideal for easier visualization of eye formation.

Note: This protocol utilizes the Wnt inhibitor *N*-((5-chloro-8-hydroxyquinolin-7-yl)(4-(diethylamino)phenyl)-methyl)butyramide (CBA-1) (Zhang et al, 2020) but can be used to examine Wnt inhibition and *in vivo* toxicity of any compound. Doses given to animals may need to be adjusted based on potency and toxicity of compounds.

Note: A subset of the imaging for this protocol was done using the Vertebrate Automated Screening Technology (VAST) Bioimager (Union Biometrica; Pardo-Martin et al., 2010; Chang et al., 2012; Pulak, 2016). Using the VAST Bioimager allows for efficient high-throughput larval imaging with automatic positioning of zebrafish larvae; however, imaging may also be done on any fluorescence-equipped stereo microscope with a camera and imaging software.



Note: All procedures described in this protocol have been approved by the University of Kentucky's Institutional Animal Care and Use Committee (protocol 2015-2225). All zebrafish experiments that are performed following this protocol must be approved by the user's Institutional Animal Care and Use Committee. The eye regeneration assay may not require approval at some universities, as the use of zebrafish <3 days post-fertilization (dpf) is not regulated under current animal research guidelines.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
BIO	Sigma-Aldrich	B1686-5MG
CaCl	VWR	Cat #: 1B1110-500G
CBA-1 (biotinylated variant of N-((5-chloro-8-hydroxyquinolin-7-yl)(4-(diethylamino)phenyl)-methyl)butyramide)	Zhang et al 2020	N/A
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	D2438-50ML
KCl	VWR	Cat #: 0395-500G
MgSO ₄	Sigma-Aldrich	Cat #: 230391-500G
NaCl	VWR	Cat #: BDH9286-500G
Tricaine-S	Pentair Aquatic	TRS1
XAV939	Sigma-Aldrich	X3004-5MG
Experimental models: Organisms/strains		
CG1 zebrafish line	Mizgireuv and Revskov, 2006	N/A
Tg(6xTCF/LEF-miniP:dGFP) transgenic zebrafish line	Shimizu et al, 2012	N/A
Software and algorithms		
Fiji ImageJ	ImageJ	https://imagej.net/Fiji/Downloads
Fluorescence-Equipped Stereo Microscope Imaging System	Nikon	SMZ 25 C-LEDS/ No. 222142
Stereo dissection microscope	Nikon	SMZ 745
VAST BioImager	Union Biometrica	N/A

MATERIALS AND EQUIPMENT

50× E3 Media		
Reagent	Final concentration	Amount
NaCl	250 mM	14.6 g
KCl	8.5 mM	0.65 g
CaCl	16.5 mM	2.20 g
MgSO ₄	16.5 mM	4.05 g
Double-Distilled H ₂ O	n/a	1 L
Total	n/a	1 L

1× E3 Media – 20 mL 50× E3 Media in 980 mL H₂O

Adjust the pH of 1x E3 media to 7.0 with NaHCO₃ powder.

Tricaine-S Solution – 4 g Tricaine-S/1 L reverse osmosis (RO) H₂O

Buffer solution to pH 7.0 using NaHCO₃ powder.

Note: 50× E3 Media is suitable for use at ~20°C within 1 month. 1× E3 Media is suitable for use at ~20°C within 2 weeks. Tricaine-S Solution is stored at 4°C and suitable for use within 1 month.

Drug solutions: Drugs are prepared at a 10mM stock concentration by dissolving powder in an appropriate volume of DMSO. From the stock, drugs are then dissolved in 1×E3 media or fish system water to a final concentration of 1–10 μM (depending on the experiment being performed).

STEP-BY-STEP METHOD DETAILS

Wnt inhibitor drug screen in 6xTCF/LEF:GFP zebrafish

⌚ Timing: 5 days

Note: GFP signaling in 6xTCF/LEF-miniP:dGFP transgenic zebrafish is in response to induction of Wnt signaling during normal development. In this assay, chemical inhibition of the Wnt pathway will lead to a decrease in GFP fluorescence, signifying a decrease in Wnt pathway signaling.

Day 1

1. Prepare 1×E3 media and tricaine solution according to the materials table.
2. Set up an incross of 6xTCF/LEF-miniP:dGFP transgenic zebrafish for breeding. To do so, a 3:2 ratio of females to males should be set up in a breeding tank with shallow water.

Note: A total of 12 embryos are used per drug treatment (1 row of a 96-well plate). For example, with vehicle control, XAV939 and BIO controls, and one drug of interest, a total of 48 GFP embryos will be required. Since not all embryos will be GFP-positive or survive, aim to have >100 embryos to collect by setting up ~4 mating tanks of zebrafish.

Day 2

3. Collect embryos in a 10 cm² petri dish with approximately 25 mL of 1×E3 media and place in a 28°C incubator overnight. Keep ~30 embryos per petri dish.

Day 3

4. At 24 hpf, screen 6xTCF/LEF-miniP:dGFP transgenic zebrafish embryos for GFP fluorescence using a fluorescence-equipped stereo microscope. Remove any embryos that are not fluorescent.

Note: GFP fluorescence will present in a fan-like shape around the tail and bilateral sections in the head at this timepoint in development (Figure 1A).

⚠ **CRITICAL:** It is important to choose embryos of similar fluorescence intensity and avoid outliers. Select larvae with equal GFP expression to use for the drug screen. Remove any larvae that are not fluorescent or have a different fluorescence intensity than the surrounding larvae (Figure 1B). Approximately 60% of embryos collected will meet screening criteria and can be used for drug screening.

5. Under a dissecting microscope, dechorionated screened larvae using ultra-fine point forceps to gently pull apart the chorion and release the embryos.

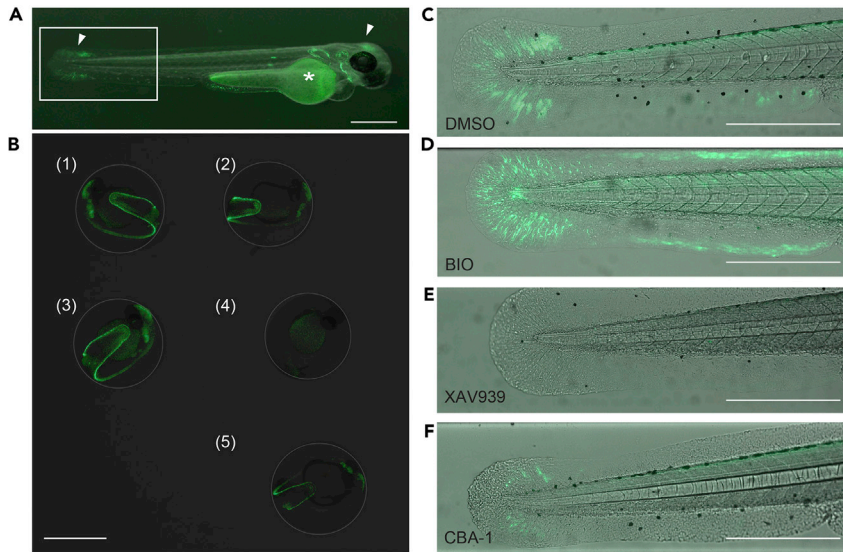


Figure 1. 6xTCF/LEF-miniP:dGFP Wnt inhibitor drug screen

(A) 6xTCF/LEF-miniP:dGFP zebrafish at 48 hpf; GFP fluorescence is an indicator of Wnt signaling during normal development. There is extensive TCF/LEF-driven GFP expression in the head and tail regions (arrowheads). The yolk is auto-fluorescent (*). The white box shows the tail area imaged for drug screen in (C-F).

(B) Screening of 6xTCF/LEF-miniP:dGFP zebrafish at 24 hpf. The chorion is circled to make visualization easier. Embryos numbered 1-3 are examples of fish that have similar, bright fluorescence and would be selected for the drug screen. Embryo 4 has no GFP fluorescence, while embryo 5 has very dim fluorescence; both should be excluded from the screen.

(C) DMSO (vehicle) control fish showing normal pattern of GFP expression in the tail.

(D) Treatment with the Wnt pathway activator BIO leads to an increase in GFP fluorescence in the tail.

(E) Treatment with the Wnt inhibitor XAV939 leads to a decrease or complete loss of GFP fluorescence in the tail.

(F) Treatment with a less potent Wnt pathway inhibitor, CBA-1, leads to a decrease but not complete loss of GFP fluorescence. Images (C-F) were taken at 72 hpf. Scale bar = 500 μm

Note: Dechoriation can also be performed using Pronase at a final concentration of 1 mg/mL in 1x E3 media.

6. In a flat-bottom 96-well plate, add 1 dechorionated fish with 100 μL E3 media to each well.
 - a. Replace 1x E3 media in the plate of embryos with fresh 1x E3 media
 - b. Cut the tip off of a 200 μL pipette tip, just large enough for a 2 dpf zebrafish larva to fit through. Using a P200 pipette, aspirate 100 μL E3 media with one zebrafish larva from the plate and add to an empty well.
7. Add 100 μL of 2 \times concentration of the desired drug (see note below) to each well, respectively. Do the same dilution for DMSO/vehicle in E3 media as a control for normalization of fluorescent signal intensity.

Note: For this protocol, 10 μM of the Wnt pathway inhibitor CBA-1 was used. 1 μM BIO, a GSK3 inhibitor that activates Wnt pathway signaling, was used as a positive control (Sato et al., 2004). 1 μM XAV939, a Tankyrase 1 inhibitor that inhibits Wnt pathway signaling, was used as a negative control (Huang et al, 2009). DMSO was used as a vehicle control at a final concentration of 0.1%.

Note: This addition of 100 μL of 2 \times drug + 100 μL E3 media will bring the final drug concentration to 1 \times . From the 10 mM stock solutions, CBA-1 and DMSO/vehicle control were diluted 1:500 in E3 media for the 2 \times concentration and BIO and XAV939 were diluted 1:5,000 in E3 media.

8. Place the plate into a 28°C incubator and leave for 48 h of drug treatment.

Note: If the drug of choice is light-sensitive, incubation can be done in the dark (either in an incubator in a dark room or by covering the plates with aluminum foil).

Day 4

9. Examine the larvae daily and remove any larvae that are dead or developmentally abnormal. The drug may be too toxic if there are a large number of dead or deformed larvae. Record the number of dead/deformed larvae for toxicity purposes.

Note: If 10 µM of the drug of choice is too toxic for zebrafish larvae or no effect is seen at this dose, the maximum tolerated dose for your drug of choice should be determined and used for the drug screen (see Troubleshooting Problem 2 below).

Day 5

10. After 2 days of drug treatment, image 72 hpf larvae on the VAST Bioimager at 488 nm excitation/ 509 nm emission to visualize GFP (Union Biometrica; [Pardo-Martin et al., 2010](#); [Chang et al., 2012](#); [Pulak, 2016](#)). Image only the tails of the *6xTCF/LEF-miniP:dGFP* larval fish just above the anal canal and avoid imaging yolk. The autofluorescence of the yolk can interfere with downstream quantification. Using the tail only for GFP-quantification yields the most reproducible results ([Figures 1C–1F](#)).

- Start with DMSO control fish to set the exposure level before moving on to positive and negative controls and then the drug(s) of choice.
- Maintain constant exposure settings throughout all images.

Note: The VAST bioimager automatically adds Tricaine-S solution to wells of 96-well plate to anesthetize animals for imaging. If not using VAST bioimager, fish can be anesthetized in a final concentration of 50–100 mg/L of Tricaine-S before imaging.

▮▮ **Pause point:** Once images have been taken, quantification can be done at any point.

11. Quantify fluorescence using FIJI Image J software and macro as previously described in [Haney et al. 2020](#).

- Multiply (mean fluorescence intensity) × (area of fluorescence) for each image.
- Normalize values to vehicle control (DMSO in this experiment) by dividing the above number by the average (mean × area) for all DMSO fish.

Caudal fin regeneration assay in wild-type fish

⌚ **Timing:** 5 days

Note: Zebrafish caudal fins will regenerate if they are cut immediately rostral to the bifurcation of the caudal fin ([Figure 2A](#)). This regeneration is dependent on Wnt pathway signaling ([Stoick-Cooper et al., 2007](#)). Chemical inhibition of Wnt signaling will lead to reduced caudal fin regeneration, signifying a decrease in Wnt pathway signaling.

Note: For this assay you will need 6 adult wild-type zebrafish (of any sex) per drug treatment. For example, if using DMSO/vehicle control, XAV939 control, and a potential Wnt inhibitor compound, a total of 18 adult zebrafish would be required for this experiment. This number was chosen as a pilot study to not overcrowd tanks and not require too much

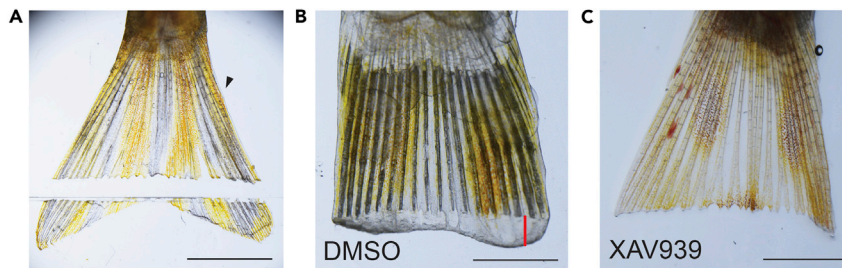


Figure 2. Caudal fin regeneration assay

(A) Caudal fins are amputated with a razor blade immediately rostral to the fin bifurcation. Cuts made too close to the tail musculature (arrowhead) will likely result in zebrafish mortality.
 (B) DMSO control treated zebrafish show regeneration of the caudal fin 4 days post-amputation, with the amount of growth measured (red line).
 (C) Wnt inhibitor (XAV939) treated caudal fins fail to regenerate. Scale bar = 2 mm.

drug. Power analysis can be done based of this study to decide how many fish are required for further studies.

Day 1

12. Anesthetize adult (3–12 months of age) wild-type fish by placing the fish in a 10 cm² petri dish containing 25 mL of fish system water with 100 μL of 4mg/mL Tricaine-S solution for approximately 1–2 min.
13. Remove the fish from the Tricaine-water and place on a clean 10 cm² petri dish. Using a razor blade, cut off the end of the fish's tail just rostral to the bifurcation of the caudal fin (Figure 2A).

△ CRITICAL: Do not cut the tail fin too close to the musculature in the tail (Figure 2A). Larger wounds have more blood loss and risk of infection, increasing the likelihood of mortality.

14. Immediately place fish into a large (~4L) tank with fish system water to recover for 20 min.
 - a. Repeat steps 8 and 9 for enough fish for n=6/drug treatment group.
15. After the recovery period, place amputated fish (n=6/group) in a small tank, such as a small breeding tank (18 cm × 8 cm × 7 cm), with 250 mL of fish system water (approximately 2.5 cm depth) containing 5 μM of Wnt inhibitor compound to be tested, DMSO (or the correspondent vehicle) as a negative control, or 1 μM of XAV939 as a positive control.

Note: 5 μM drug concentration was chosen for this assay due to the larger amount of drug stock required to perform this assay on adult zebrafish.

16. Leave the tank of drug-treated, amputated fish at ambient temperature (approximately 28°C). Do not feed the fish until immediately prior to water changes to keep the tank clean.

Day 2 (1 day post-amputation)

17. Move fish to a new tank with 250 mL of fresh fish system water containing the fresh drug.

Day 3 (2 days post-amputation)

18. Feed fish with dry food, wait 15 min for the fish to eat, and then move fish to a new tank with 250 mL of fresh fish system water containing the fresh drug.

Day 4 (3 days post-amputation)

19. Move fish to a new tank with 250 mL of fresh fish system water containing the fresh drug.

Day 5 (4 days post-amputation)

20. Image fish at 4 days post-amputation using any stereo microscope. Take images of the tail regeneration front (Figure 2B and C). Fish can be anesthetized with a final concentration of 50–100 mg/L or euthanized with >400 mg/L Tricaine-S for ease of imaging.
21. Using the measurement feature in FIJI Image J software, measure the length of growth of each tail from the cut point to the farthest point of growth (Figures 2B and 2C).

Note: Fish may be fixed in 4% paraformaldehyde in PBS overnight at 4°C then moved to PBS and held in a 4°C refrigerator for imaging within one week.

Eye rescue in wild-type fish

⌚ Timing: 4 days

Note: Zebrafish eye development is dependent on a proper balance of Wnt pathway signaling. When the balance of Wnt/Beta-catenin signaling is altered towards increased Wnt signaling during early development, zebrafish will develop an eyeless phenotype (van de Water et al, 2001). In this assay, over-stimulation of Wnt pathway signaling is achieved by treating zebrafish embryos during the first 6 h of development with the compound BIO. The resulting eyeless phenotype can then be rescued by reducing Wnt pathway signaling via treatment with a Wnt pathway inhibitor compound.

Note: This experiment requires 30 embryos per drug treatment for a pilot study. For this experiment, with BIO and DMSO/vehicle control, XAV939 and a Wnt inhibitor, a total of 120 embryos are required. This pilot experiment can then be repeated with 3 independent replicates and the results between trials averaged to increase statistical power.

Day 1

22. Set up an incross of any pigmented wild-type zebrafish line for breeding. To do so, place a divider in a breeding tank with shallow water. A 3:2 ratio of females to males should be set up, using the divider to separate males and females.

Day 2

23. In the early morning, remove the divider from the breeding tank(s) to allow the zebrafish to begin breeding. Check for embryos every 15 min after removing the divider and collect any embryos that have been laid, recording the time of collection as time gates were pulled. This can be repeated, checking for new eggs laid and collecting them every 15 min until enough eggs are collected to perform the experiment.
24. Collect embryos in a 10 cm² petri dish in 1x E3 media and place in a 28°C incubator until ready to use. Embryos will be used for drug-treatment at 6 hpf.
25. In a 6-well plate, add 1 μM BIO + 10 μM CBA-1, or experimental drug of interest, in 9 mL E3 media per well.
 - a. Use vehicle/DMSO, BIO treatment alone, and BIO plus 10 μM XAV as controls.
26. Add 6 hpf embryos into the plate with 20 fish/well using a transfer pipette with as little volume of liquid as possible.

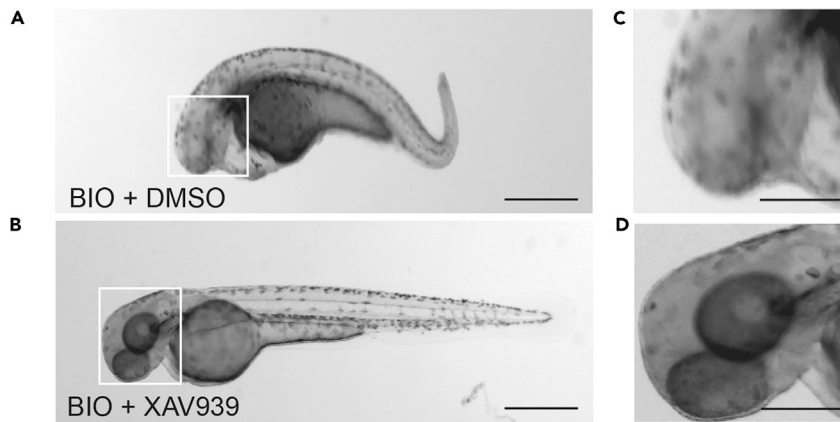


Figure 3. Eye rescue assay

(A) Treatment with BIO leads to overactivation of Wnt signaling and a loss of eye development, as well as other developmental abnormalities.
 (B) Simultaneous addition of a Wnt inhibitor (XAV939) along with BIO treatment rescues the eyeless phenotype.
 (C and D) show the boxed head regions from A and B, respectively. Scale bar in A and B = 500 μm . Scale bar in C and D = 250 μm .

Note: When transferring embryos to the drug-treatment wells from the 10 cm^2 petri dish they were collected into, make sure to add as little E3 media with the embryos as possible. This ensures the drug concentration remains at the desired concentration. Hold the transfer pipette vertically and allow embryos to settle to the bottom of the transfer pipette before expelling fish to avoid transferring large volumes of liquid.

△ CRITICAL: Make sure fish are set up to breed with gates in place and keep track of the time the fish lay embryos. The addition of drug at the shield stage of development (at 6 hpf) is critical for viable embryo development and the eyeless phenotype.

27. Place embryos in drug treatment in a 28°C incubator.

Day 4

28. Using a stereo microscope, count the number of eyes present in fish at 48 hpf (Figures 3A–3D). Record the number of fish with no eyes, one eye, or two eyes.

Note: Eye development can be assessed at 24 or 48 hpf depending on toxicity of the drugs being used. The phenotype will not change after 24 hpf, however the eyes will be larger and more pigmented at 48 hpf and are easier for counting and imaging.

Note: Treatment with BIO is toxic to zebrafish embryos during development as it interferes with normal early development pathway signaling. Phenotypes such as pericardial enlargement, pink coloration in the yolk, and tail atrophy or curvature may be seen. These phenotypes will not affect the eye rescue phenotype outcomes.

Note: A dose response curve can be done to determine strength of Wnt pathway inhibition. This is done by treating groups of embryos with 1, 2, 5, and 10 μM of the drug of interest plus 1 μM BIO in the same manner as described above. Results should indicate an increase in the number of two-eyed embryos, a decrease to almost no one-eyed embryos, and a lack of no eyed embryos as the dose increases.

EXPECTED OUTCOMES

For the drug screen in *6xTCF/LEF-miniP:dGFP* transgenic animals, any drugs that inhibit Wnt pathway signaling should lead to a decrease in GFP fluorescence in the tail region. For very good inhibitors, a complete loss of GFP fluorescence may be seen in the tail region after 48 h of drug treatment (72 hpf). For the controls, BIO treatment will cause an increase in GFP expression and XAV939 will cause almost a complete loss of GFP expression (Figure 1D and E). For less strong Wnt pathway inhibitors, there will be a decrease in GFP expression that can be seen and quantified, but not a complete loss of GFP signal (Figure 1F).

For the caudal fin regeneration assay, zebrafish caudal fins will regenerate if they are cut just rostral to the bifurcation of the fin. This regeneration is dependent on Wnt signaling. In this experiment, DMSO serves as the vehicle control and XAV939, a known Wnt pathway inhibitor, serves as the positive control. The fish in the DMSO/vehicle control group will have normal Wnt pathway signaling, and will have measurable tail fin regeneration after 4 days at the site of amputation (Figure 2B). Inhibition of Wnt signaling with XAV939 will lead to no tail fin re-growth where the fin was cut (Figure 2C). The effects of the small molecule being tested should be compared to those of the DMSO/vehicle control group and the XAV939 positive control group. If the small molecule being tested is a strong inhibitor of Wnt signaling, regrowth of the tail fin will be significantly reduced compared to DMSO.

Normal development of the zebrafish embryo is dependent on the correct balance of Wnt pathway signaling. Eye development is especially sensitive to disruptions in timing of Wnt signaling events. Addition of BIO to embryos at 6 hpf (shield stage) activates Wnt signaling and results in a failure of eye development (Figures 3A and 3C). However, when embryos are concurrently treated with BIO and a Wnt inhibitor, the balance of Wnt pathway signaling will be restored and the loss of eye phenotype is reversed (Figures 3B and 3D).

LIMITATIONS

There is an inherent degree of variation that should be expected in animal studies. A large enough sample size needs to be used to ensure enough statistical power to see differences in GFP expression, fin regeneration, and eye development. Drug concentrations and numbers of animals used may need to be optimized based on the properties of the drug and how well the imaging set-up used can distinguish changes in GFP fluorescence. Another potential limitation is that the drug needs to be soluble in water and stable enough to be absorbed from the water into the zebrafish larvae over the treatment period without breaking down. Additionally, although rare, some compounds may be toxic to zebrafish larvae but not in mammals, so false positives for toxicity may be observed. The opposite may also be true, with some drugs being toxic in mammals but not in the zebrafish, resulting in false positives for drug efficacy.

TROUBLESHOOTING

Problem 1

The drug of interest precipitates in water (protocol steps 7, 15, and 25)

Potential solution

Check the drug properties to make sure that the drug is soluble in water or at least will not precipitate out at the desired concentration. A lower concentration can be tried to see if the drug still precipitates out. However, some drugs will continue to precipitate and cannot be used for zebrafish assays *in vivo* as the zebrafish larvae may consume the drug precipitates.

Problem 2

The drug of choice is too toxic and kills the fish at the suggested concentration, or there is no effect seen with the dose of the drug chosen (protocol step 9).

Potential solution

A maximum tolerated dose (MTD) assay ([Hutchinson et al, 2009](#); [Maes et al, 2012](#)) can be done to determine what the MTD is for your drug of choice in zebrafish. This is done by testing a broad range of doses in zebrafish larvae at the same age that would be done in the experiment, and quantifying signs of obvious toxicity or animal death. The highest dose that is tolerated without causing toxicity or death should be used for animal studies. This dose may be different in larval and adult fish.

Problem 3

In the TCF/LEF larval drug screen, the entire fish appears fluorescent in the GFP channel when treated with the Wnt inhibitor of choice (protocol step 10).

Potential solution

Some drugs have auto-fluorescent or fluorescent properties in the GFP channel. Check to make sure the drug of choice does not fluoresce in the GFP channel. If so, this assay cannot be performed with this drug; however the other two assays can still be done to assess Wnt inhibition *in vivo*.

Problem 4

When performing the tail regeneration experiment, the tail fails to grow back in the DMSO/vehicle control group (protocol step 13).

Potential solution

It is likely that the cut made in the tail was too far rostral in the tail and into the tail musculature. Review [Figure 2A](#) for a clear view of the optimal cutting location.

Problem 5

When performing the eye rescue experiment, the eyes fail to disappear with BIO treatment (protocol step 26).

Potential solution

It is likely that the BIO was not added at the correct timepoint before eye development began. Begin timing immediately after gates are pulled, restarting the timer and collecting embryos every 15 min, so that BIO is added during the shield stage of development (6 hpf). Embryos should also be kept at 28°C so that the speed of development is not changed due to a non-physiologic temperature.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jessica Blackburn at jsblackburn@uky.edu.

Materials availability

Zebrafish lines used in this study are available via the Zebrafish International Research Center (ZIRC) and can be made available by request. Plasmids used to create zebrafish lines or zebrafish themselves can also be shipped upon request to the lead contact.

Data and code availability

This study did not generate/analyze any datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.G.H., J.S.B., and C.L.; investigation, M.G.H.; writing – original draft, M.W. and M.G.H.; writing – review & editing, M.W., M.G.H., J.S.B., and C.L.; funding acquisition, M.G.H. and J.S.B.; supervision, J.S.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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