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Notochord Injury Assays that Stimulate Transcriptional Responses in Zebrafish Larvae

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14   **[Abstract]** Zebrafish have become an increasingly important model organism in the field of wound

15   healing and regenerative medicine, due to their high regenerative capacity coupled with high-resolution

16   imaging in living animals. In a recent study, we described multiple physical and chemical methods to

17   induce notochord injury that led to highly specific transcriptional responses in notochord cellular

18   subpopulations. The notochord is a critical embryonic structure that functions to shape and pattern the

19   vertebrae and spinal column. Here, we describe precision needle injury, tail-notochord amputation, and

20   chemical inhibition of caveolin that trigger a wound-specific *wt1b* expression response in the notochord

21   sheath cell subpopulation. We propose that these procedures can be used to study distinct cell

22   populations that make up the cellular processes of notochord repair.

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24   **Keywords:** Zebrafish, Notochord, Tail fin, Injury, Amputation, Tissue repair, Tungsten wire, Nystatin

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26   **[Background]** The notochord is a transient embryonic structure that provides axial support and

27   signalling information to the developing embryo (Ellis *et al.*, 2013). It is comprised of two structurally

28   distinct cell populations: the inner vacuolated cells that provide embryo support and structure, and the

29   outer sheath cells that maintain turgor pressure for the vacuolated cells as well as patterning the

30   developing vertebrate spine (Wopat *et al.*, 2018; Lleras Forero *et al.*, 2018; Figure 1A). We have recently

31   discovered that *wilms' tumour 1b* (*wt1b*) is specifically expressed in a notochord sheath cell

32   subpopulation that emerges at the site of damage and is maintained throughout repair and formation of

33   adult vertebra structure in zebrafish (Figures 1B and 1C; Lopez-Baez *et al.*, 2018). WT1 is a zinc-finger

34   transcription factor involved in mesodermal tissue development, adult tissue homeostasis, and becomes

35   reactivated during epicardial tissue damage (Hastie *et al.*, 2017). Our discovery that *wt1b* becomes

36   expressed at the notochord wound may have important implications for the development of therapies

37   for vertebrae spinal injuries or degenerative processes.

38   In zebrafish wounding and regeneration models, injury is induced by a variety of methods such as

39   amputation, surgical resection, irradiation, laser ablation and genetic ablation (Gemberling *et al.*, 2013).

For example, in larval zebrafish, syringe needles of various sizes have been used for tail fin amputation and spinal cord injury experiments (Lisse *et al.*, 2015; Wehner *et al.*, 2017).

We have conducted notochord injury assays in zebrafish larvae using physical and chemical approaches (Lopez-Baez *et al.*, 2018). Electrolysis-sharpened tungsten wire and insect pins described here and in our recent paper induce precise, localized injury and trigger wound-specific *wt1b* expression (Figures 1B and 1C). The structural integrity of the notochord can also be disrupted chemically by treating embryos with nystatin, a small molecule which binds sterols and disassembles caveolae (Rothberg *et al.*, 1992), which are particularly abundant in the notochord (Lim *et al.*, 2017). We detected increased *wt1b* expression in nystatin treated notochords suggesting changes in caveolae caused by non-physical damage and stress may also induce *wt1b* expression.
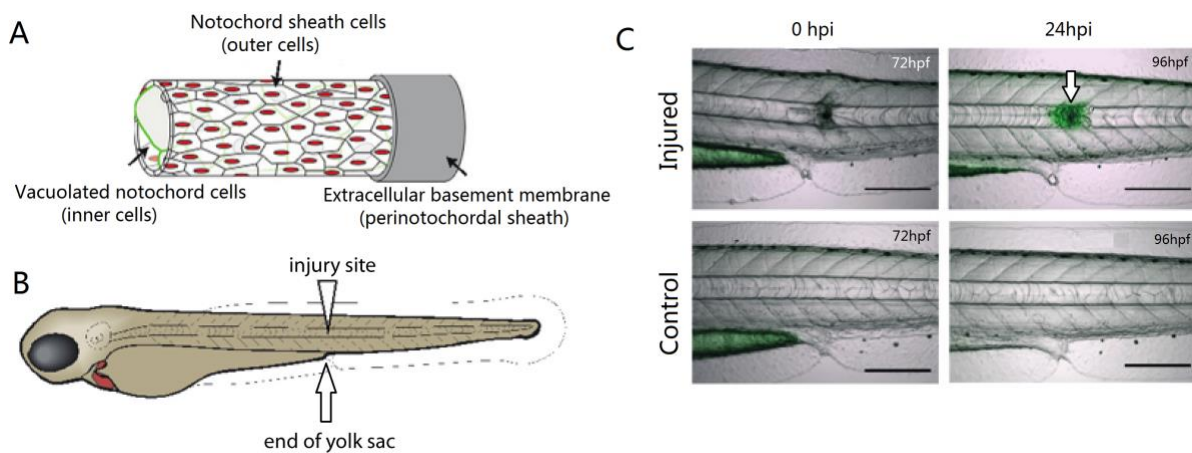
Figure 1. Cell populations of the notochord and the *wt1b* notochord wound response.
A. Schematic of the cell populations of the notochord. The notochord is comprised of two physically distinct cell populations: an epithelial-like notochord sheath cell population (outer cells; red) and a large vacuolated notochord cell population (inner cells, green)*,* which are tightly wrapped by a thick, elastic extracellular basement membrane (peri-notochordal sheath). B. Schematic of the zebrafish embryo and the site of the notochord wound at the end of the yolk sac (YS). C. Needle injury triggers localised *wt1b:gfp* expression in the notochord at the site of damage by 24 h post injury (hpi; arrow). Scale bars = 100 μm in Panel C.

Materials and Reagents

1. Ø 0.25 mm Tungsten wire (Alfa Aesar, catalog number: 010073.G2)
2. Metal needle holder (VWR International Ltd. UK, catalog number: MURRL110/01)
3. Sterile scalpel blade (Swann Morton UK, catalog number: 11708353)
4. 0.10 mm Austerlitz insect pins, stainless steel (Fine Science Tools, catalog number: 26002-10)
5. Glass Pasteur Pipettes length: 145 mm (Brand, catalog number: 7477 15)
6. Petri dish (Thermo Scientific, catalog number: 15370366)

7. Zebrafish larvae (3-7days post fertilization)

Non-pigmented *mitfa* mutant (*nacre* allele) (Lister *et al.*, 1999) may be preferable for ease of imaging. For our experiments, we used the Tg(R2*col2a1a:mCherry*) transgenic line to visualise notochord sheath cells (Dale and Topczewski, 2011), and the Tg(*wt1b:gfp*) line to study the wound response in the notochord (Perner *et al.*, 2007).

Important: Zebrafish older than 5 days post fertilization are protected animals by UK and EU law, and require proper animal procedure licenses and approval from institutional ethics committees.

8. Dimethylsulfoxide (DMSO) (Sigma-Aldrich, catalog number: D2650-100ML)

9. Nystatin (Sigma-Aldrich, catalog number: N6261-500KU)

10. Agarose (Invitrogen, catalog number: 15510-027)

11. Tricaine (MS-222, 3-amino benzoic acid ethyl ester, Sigma, catalog number: A-5040)

12. NaOH pellets (Sigma, catalog number: S8045-500G)

13. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653-1KG)

14. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9333-500G)

15. Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) (Sigma-Aldrich, catalog number: 223506-500G)

16. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) (Sigma-Aldrich, catalog number: M2393-500G)

17. E3 embryo medium (60x stock solution) (see Recipes)

18. Tricaine (MS-222) (1x working solution) (see Recipes)

19. NaOH (5 M) (see Recipes)

Equipment

1. Microscopes:

Upright Stereomicroscope (*e.g.*, Nikon, model: SM2645)

Light microscope (*e.g.*, Olympus, model: SZX16)

Confocal microscope (*e.g.*, Nikon, model: A1R)

2. Electrolysis device for tungsten wire sharpening (the device shown in this protocol is custom-made and no longer in production; Figure 2A)

However, users can set up their own equipment.

1) Parts required are: a). DC power supply (3-20V, such as Bosch C3 smart car battery charger, Part No: 0092C35000), b). carbon electrode rod (Lasec, Catalog number: ERDI9470), c). crocodile clips (optional, DC power supply may come with leads with crocodile clips), d) a 200ml glass container (jar) with lid.

2) Assemble the equipment: Plug the leads into the charger. Using crocodile clips, connect the carbon electrode rod to the negative terminal (black lead), connect the metal holder to the positive terminal (red lead). Set the voltage to 6V and fill up the jar with 100ml of NaOH solution. The equipment is now ready for use.
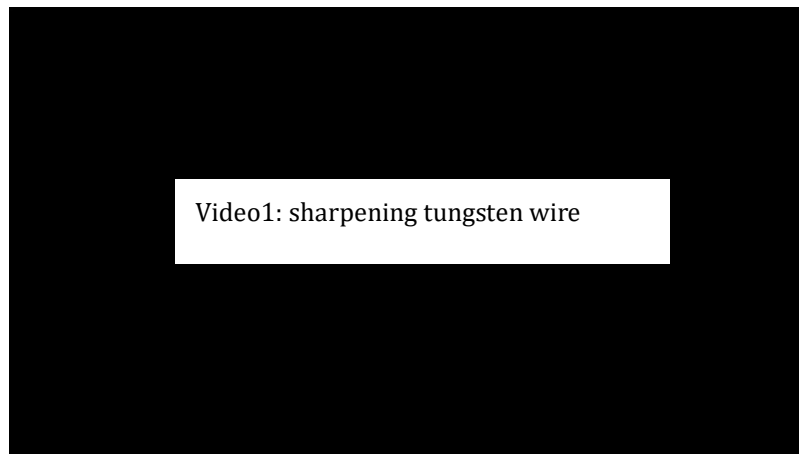
3. Bunsen burner
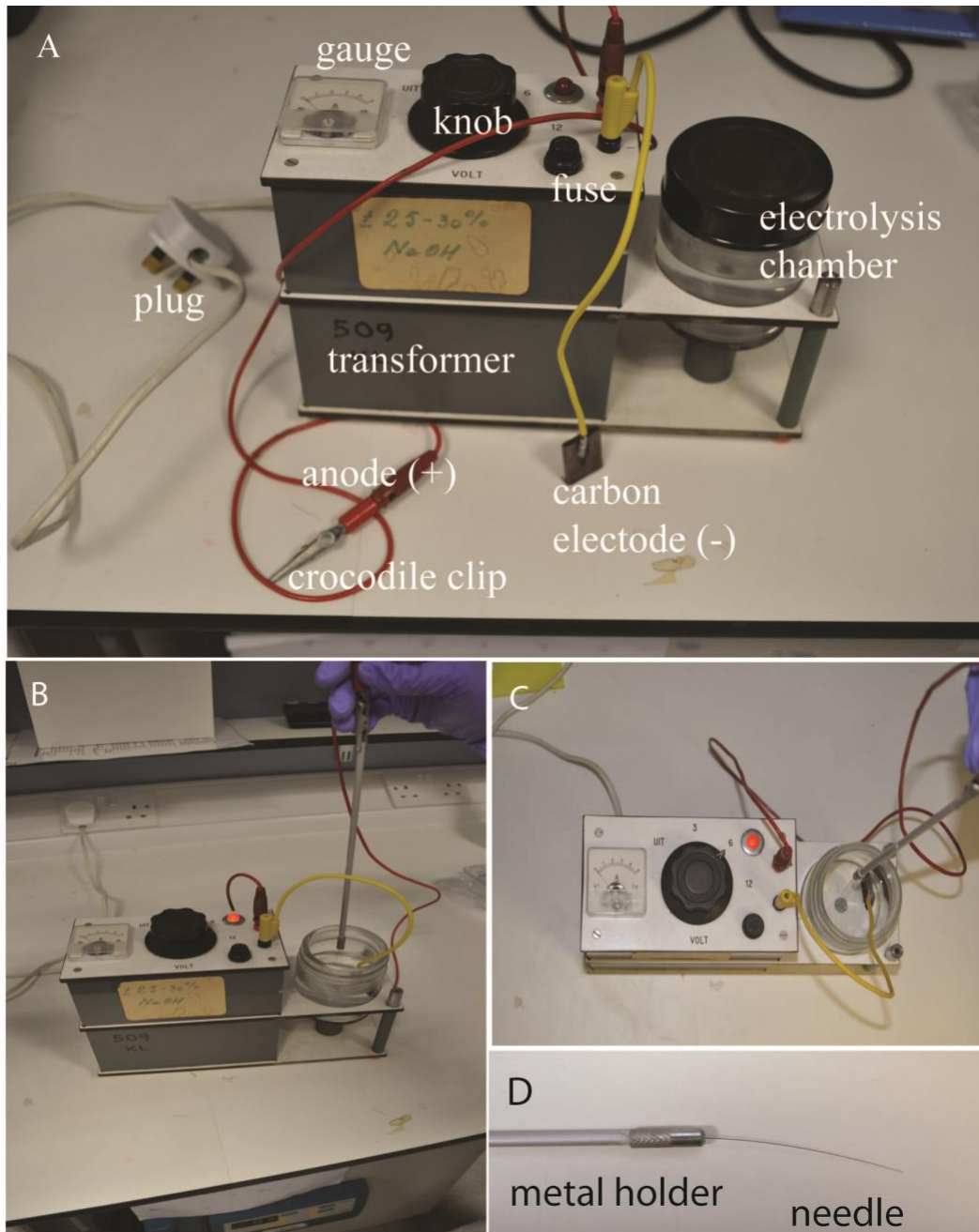
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Figure 2. Preparation of electrolysis-sharpened needle. A. Electrolysis-based apparatus, consisting of a rectified DC transformer, an anode with crocodile clip (red), a cathode with carbon electrode (yellow), electrolysis chamber with 5M NaOH electrolyte. B-C. Lateral and dorsal views of a tungsten wire needle being sharpened. With the power on (6 V), the mounted needle is held vertically and dipped into and out of the electrolyte steadily and slowly until the desired tip is achieved. D. A finished electrolysis-sharpened tungsten wire needle.

Procedure

A. Notochord needle injury of zebrafish larvae

1. Prepare electrolysis-sharpened tungsten wire (adapted from Brady, 1965) (Figure 2).

   a. Cut off 3 cm of tungsten wire and mount it into a needle holder

   b. Connect the metal handle of the needle holder to the (+) terminal of the transformer using a crocodile clip, connect the cathode with carbon plate to the (-) terminal.

   c. Place the carbon electrode in the glass chamber with 100 ml of 5 M NaOH solution and switch on the transformer, set the output voltage to 6 V. With the mounted needle held vertically, dip the needle into and out of NaOH, slowly and steadily until desired tip is produced. Faster movement = Longer slope on needle, Slower movement = Shorter tip with more angled slope. The dial gauge of current reads between 0 and 1 A when the needle moves up and down. It takes about 2.5 min to sharpen a needle from 0.25 mm to 0.02 mm in diameter.


Video1: sharpening tungsten wire

2. Alternatively, 0.1 mm insect pins can also be used to injure the notochord

   a. Take a clean glass pipette and using a Bunsen burner, bend the thin side in the middle in order to create a 45 degree angle. This will help with the injury manipulation procedure.

   b. Close the hole of the thin side by about three-quarters using the Bunsen burner. This is done by placing the tip in the strongest part of the flame and rotating it in a circular manner.

   c. Take the insect pin with forceps and place it in the hole, taking care that the sharp side is facing the outside.

   d. Carefully continue burning the tip of the glass pipette in the weakest part of the flame, until the hole is closed and the insect pin secure. The insect pin will burn if exposed to too much heat. Try to close the hole as much as possible before inserting the pin.
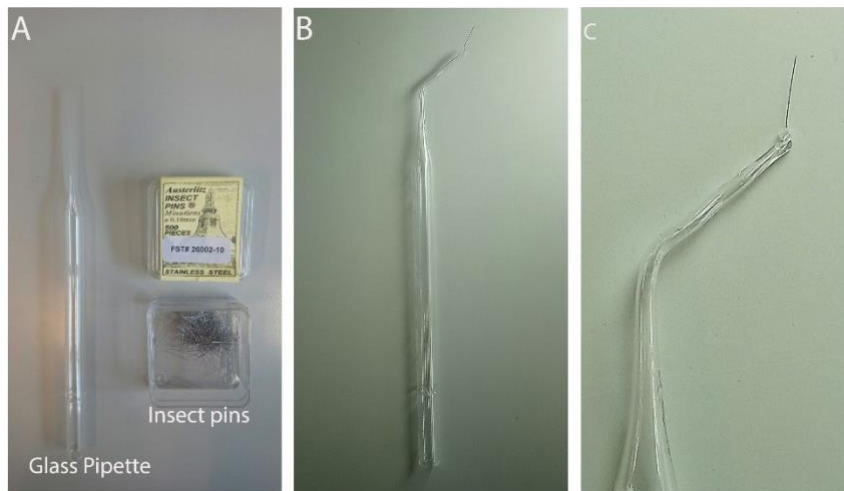
Figure 3. Preparation of insect pin. A. The required equipment. B. The finished instrument. C. A close up image of the tip.

Video2: Preparing insect pin
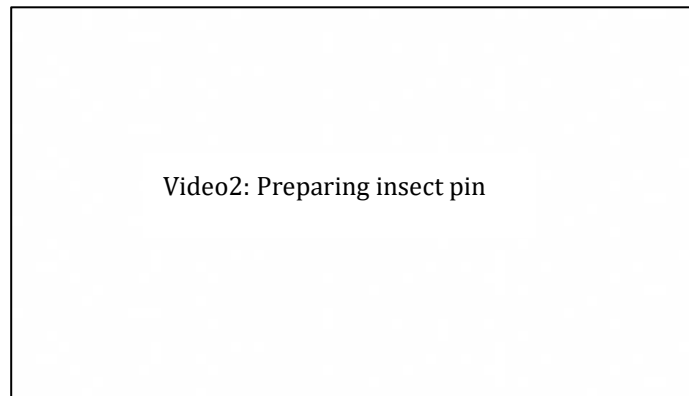
3. Prepare 1.5% agarose: Weight 1.5g agarose and melt in 100ml E3 embryo medium in a 250ml glass bottle using a microwave oven, pour a thin layer into a ϕ90mm Petri dish. About 20ml of 1.5% agarose is used. Let the agarose solidify.

4. Anaesthetize larvae in tricaine solution: Prepare 1:10,000 tricaine solution using E3 embryo medium (please see Recipes), and pour 30ml in a separate ϕ90mm Petri dish. Transfer one larva into the tricaine solution and wait until it is anesthetized.

5. Under a stereomicroscope, place one larva on it's side on a Petri dish coated with agarose so that the lateral side can be accessed with needle from above. Remove as much liquid as possible so that the surface tension adheres the larvae to the dish and prevents it from slipping. Gently insert the tip of the tungsten wire into the notochord vertically at the level of the end of the yolk sac (Figure 1B), then withdraw the wire.

6. Transfer injured larvae to a petri dish with fresh E3 medium to recover and place the dish at 28.5 °C to grow the larvae to the desired stages. Keep uninjured age-matched larvae as non-injured controls.

Video3: Notochord Needle Injury

B. Chemically-induced disruption of notochord

1. Cross fish carrying the *Tg*(*wt1b:GFP)* and the notochord-marking *Tg*(*R2-col2a1a:mCherry)* transgenes in an unpigmented *nacre-/-* background, to obtain *Tg(wt1b:GFP;R2-col2a1a:mCherry);nacre-/-* embryos.

2. Prepare fresh 5 mg/ml nystatin stock solution (5.4mM) before each use by dissolving in DMSO.

3. Dilute nystatin stock solution in E3 to obtain 20 µM final working concentration. Add this to dechorionated 48 hpf embryos in a 6-well plate. Add 0.4% DMSO to control embryos.

4. Incubate embryos at 28.5 °C for up to 48 h. After 24 hours of nystatin treatment, lesions appear along the length of the notochord. They tend to appear first in regions that are naturally compressed as the embryo moves, and then spread along the length of the notochord. The majority of embryos acquire notochord lesions, however their size and severity can be variable. Therefore, regular screening for lesions and/or the onset of *wt1b:GFP* expression is recommended in order to identify embryos with the desired level of notochord damage.

5. For imaging, anaesthetise embryos in tricaine (1:10,000), and mount sagittally in 1% low-melt agarose. Brightfield images are taken using a light microscope (Figures 4A and 4B). Expression of the *R2-col2a1a:mCherry* transgene, which marks the notochord, and the induction of the *wt1b:GFP* transgene at sites of notochord damage is visualised using confocal microscopy (Figures 4A' and 4B'').
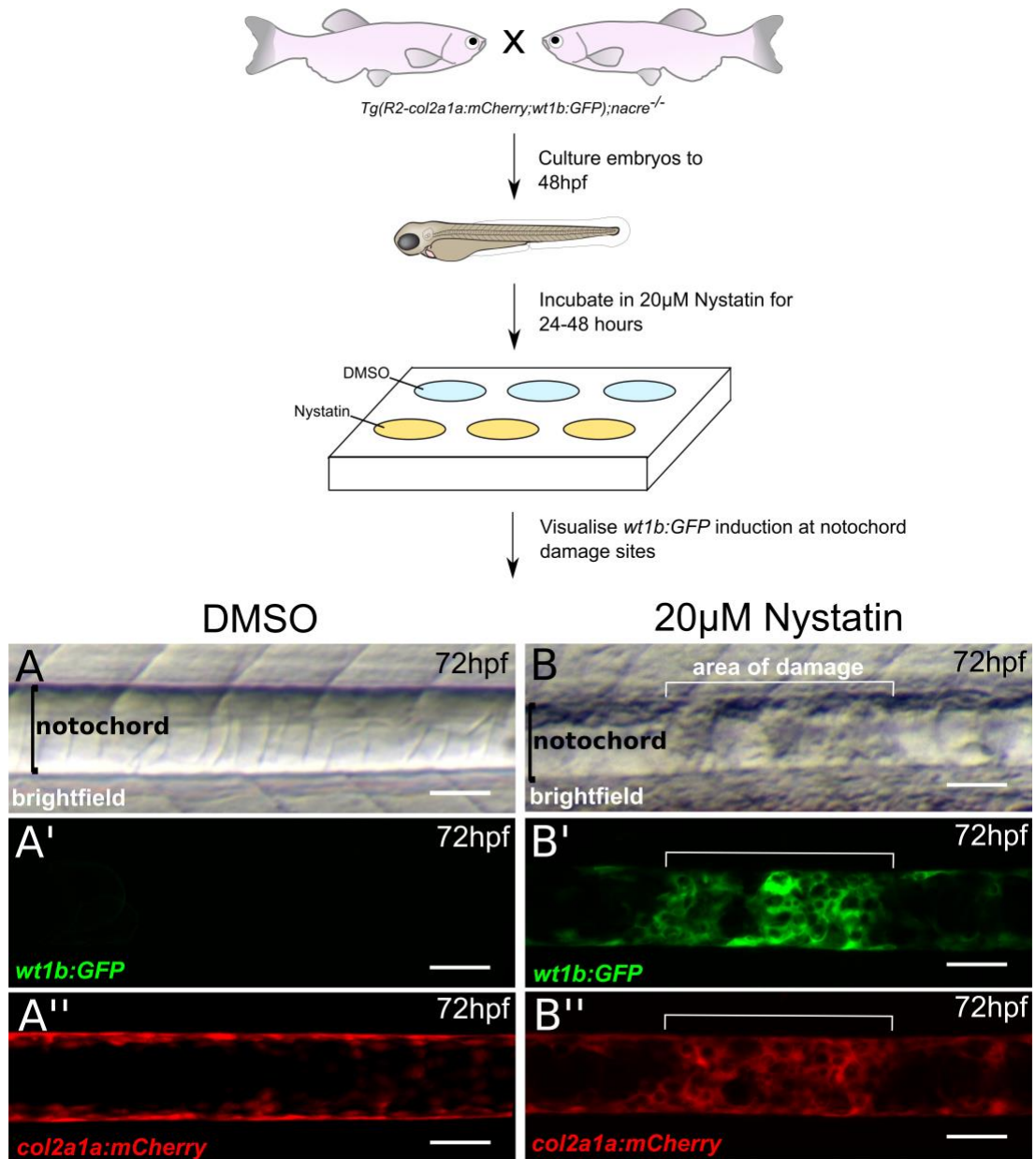
Figure 4. Disrupting notochord structure using nystatin (modified from Lopez-Baez, 2018). Nystatin is a small molecule which binds sterols and leads to the disassembly of caveolae, a component abundant in the notochord (Lim *et al.*, 2017). Tg(*wt1b:GFP;R2-col2a1a:mCherry);nacre*^{-/-} zebrafish embryos are treated with either DMSO or 20μM Nystatin from 48 hpf to 72 hpf. When observed under a light microscope, the notochord structure of (A) DMSO-treated embryos appears normal, however lesions can be observed in (B) nystatin-treated embryos. (A' and B') *wt1b:GFP* expression is induced at lesion sites, but not in control notochords. *R2-col2a1a:mCherry* expression in notochord sheath cells also shows increased cellularity at (B'') lesion sites of nystatin-treated embryos compared to (A'') DMSO controls. Scale bars are 50 μm.

C. Tail amputation

1. Prepare 1.5% agarose using E3 embryo medium and pour a thin layer into a Petri dish. Let the agarose solidify.

2. Anaesthetize larvae in tricaine solution.

3. Under a stereomicroscope, place one larva on it's side onto the solidified agarose. Remove as much as liquid as possible so the surface tension adheres the larvae to the dish and prevents it from slipping, then amputate the tail with a sterile scalpel blade with slight pressure. Amputation sites are dependent on experiments being performed (Figure 5). Amputations at the tail fin and tip of notochord site do not include notochord tissue, and do not stimulate a *wt1b:gfp* notochord injury response. Amputations beyond the tail fin and into the notochord (before caudal vein, past caudal vein) stimulate a *wt1b:gfp* expression.

4. Transfer injured larvae to a Petri dish with fresh E3 medium to recover, and place the dish at 28.5 °C to grow the larvae to the desired stages. Keep uninjured age-matched larvae as non-injured controls.
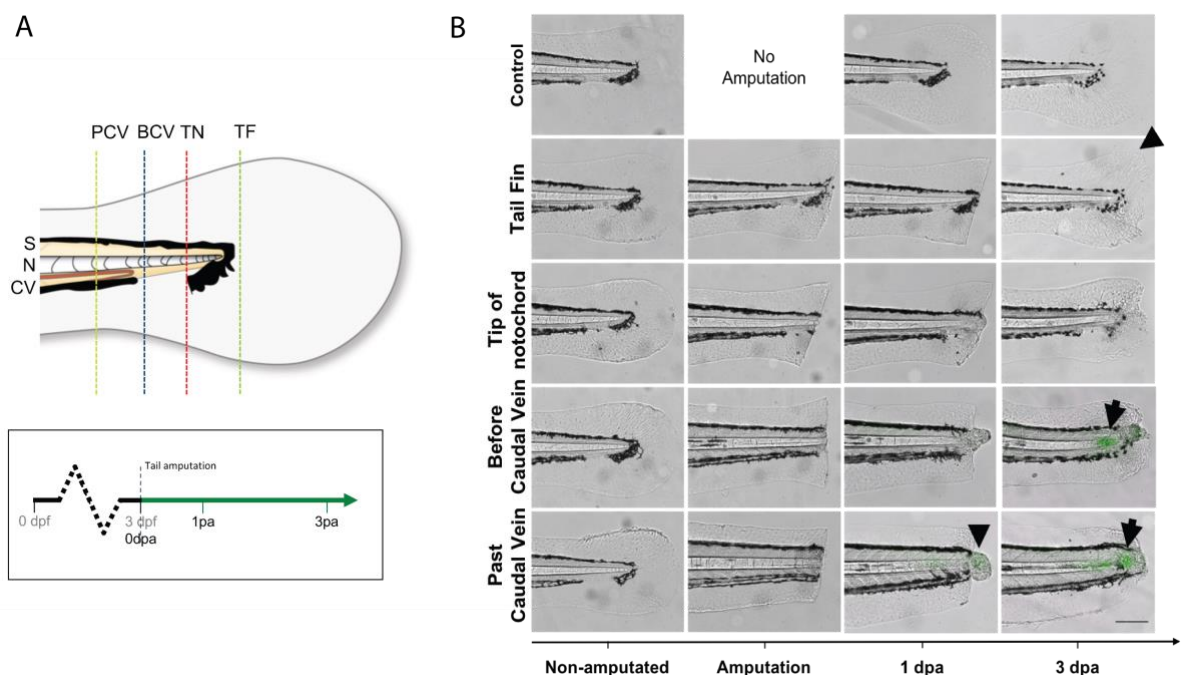
Figure 5. Selected tail amputations uncover the notochord specificity of the response (modified from Lopez-Baez, 2018). (A) Illustration of tail amputations at different tail sites and time points when images are taken. Tail fin (TF), tip of the notochord (TN), before caudal vein (BCV), past caudal vein (PCV), somite (S), notochord (N), caudal vein (CV). (B) TF and TN amputated larvae showed no GFP upregulation in their notochord after the injury, but show marked fin regeneration (arrow head). BCV and PCV amputated groups both showed strong GFP upregulations by 72hpa (arrows), with PCV amputated larvae showing an overall stronger and faster upregulation than BCV amputated larvae.

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Notes

1. In the UK and EU, all animal procedures need to be approved by the Home Office (UK) or its equivalent. Appropriate Personal Project License (PPL) and Personal individual License (PIL) are required.

2. The procedure of notochord needle injury requires a fair amount of practice, and care should be taken to not cause injury outside the needle injury site. The appearance of a small bulge structure at the site of injury within the notochord about 5 min post-surgery indicates a successful operation. It is achievable to injure 30 larvae during a period of an hour.

3. Optimising nystatin dosage for the first-time use is recommended, as there is batch-to-batch variation. A longer nystatin incubation period can be attempted however the adverse off-target effects of nystatin cause gross developmental abnormalities and embryos do not survive long-term. In our hands, treatment with 20µM nystatin from 48hpf for 24 hours gives the most consistent results. Treatment with nystatin before 48hpf is possible, however due to its off-target effects, more toxicity is seen. Embryos tolerate later nystatin treatment from 72 and 96 hpf much better, with 20µM nystatin producing notochord lesions in 60-80% of embryos after 24 hours, although these lesions are smaller in size and fewer per embryo compared those shown in Figure 4.

Recipes

1. E3 embryo medium (60x stock solution)
   17.4 g NaCl
   0.8 g KCl
   2.9 g $CaCl_2 \cdot 2H_2O$
   4.89 g $MgCl_2 \cdot 6H_2O$
   Dissolve in 1 L H_2O

2. Tricaine (MS-222) (1x working solution)
   Dissolve 0.1g of Tricaine powder in 1 L of 1x E3 medium, adjust pH to 7.0

3. NaOH (5 M)
   Dissolve 200 g NaOH pellets in 1 L H_2O

Acknowledgments

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Competing interests

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263   The authors have no competing interests.

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Ethics

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267   Procedures presented here were approved by the University of Edinburgh Ethics Committee, and

268   performed under the Home Office Project License 70/8000 to EEP at the University of Edinburgh,

269   United Kingdom; and by the Animal Experimentation Committee (DEC) of the Royal Netherlands

270   Academy of Arts and Sciences to SSM at the Hubrecht Institute and the Institute of Cardiovascular

271   Organogenesis and Regeneration WWU Münster, Germany.

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