

Video Article

A Time Differential Staining Technique Coupled with Full Bilateral Gill Denervation to Study Ionocytes in Fish

Velislava Tzaneva¹, Steve F. Perry¹¹Department of Biology, University of OttawaCorrespondence to: Velislava Tzaneva at vtzan032@uottawa.caURL: <http://www.jove.com/video/52548>DOI: [doi:10.3791/52548](https://doi.org/10.3791/52548)

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Abstract

Branchial ionocytes (ICs) are the functional units for ionic regulation in fish. In adults, they are found on the filamental and lamellar epithelia of the gill where they transport ions such as Na⁺, Cl⁻ and Ca²⁺ via a variety of ion channels, pumps and exchangers. The teleost gill is extrinsically innervated by the facial (VI), glossopharyngeal (IX) and vagus (X) nerves. The IX and X nerves are also the extrinsic source of branchial IC innervation. Here, two techniques used to study the innervation, proliferation and distribution of ICs are described: a time differential staining technique and a full bilateral gill denervation technique. Briefly, goldfish are exposed to a vital mitochondrion-specific dye (e.g., MitoTracker Red) which labels (red fluorescence) pre-existing ICs. Fish were either allowed to recover for 3 - 5 days or immediately underwent a full bilateral gill denervation. After 3 - 5 days of recovery, the gills are harvested and fixed for immunohistochemistry. The tissue is then stained with an α -5 primary antibody (targets Na⁺/K⁺ ATPase containing cells) in conjunction with a secondary antibody that labels all (both new and pre-existing) ICs green. Using confocal imaging, it was demonstrated that pre-existing ICs appear yellow (labelled with both a viable mitochondrion-specific dye and α -5) and new ICs appear green (labelled with α -5 only). Both techniques used in tandem can be applied to study the innervation, proliferation and distribution of ICs on the gill filament when fish are exposed to environmental challenges.

Video Link

The video component of this article can be found at <http://www.jove.com/video/52548/>

Introduction

ICs are the functional unit for ionic regulation in fish and are found on the epithelial surfaces of the gill filaments and lamellae^{4,6-8,10}. Although a variety of subtypes have been described that possess unique features, many of the ICs are characterized by a high density of mitochondria (thus they are also known as mitochondrion-rich cells) and/or an abundance of the enzyme Na⁺/K⁺ ATPase (NKA). Typically, these ICs house a variety of other pumps, ion channels and exchangers involved in ion regulation (e.g., Na⁺/H⁺ exchanger, Na⁺/Cl⁻ co-transporter, H⁺ pump)^{2,10,11}. The redistribution and proliferation of ICs as a compensatory mechanism is central for maintaining ion homeostasis particularly during ionic stress (e.g., exposure to ion-poor water)^{4,8,9}.

This study describes a time differential staining technique¹ to identify newly proliferated ionocytes (ICs) in fish gills. This technique is coupled with a complete bilateral denervation of the gill arches. Goldfish (*Carassius auratus*), the species used in these studies, is well suited to study proliferation of gill epithelial cells because they have a remarkable ability to structurally remodel their gills². Gill remodeling refers to the growth or retraction of an interlamellar cell mass (ILCM) when the fish (normally maintained at 15 - 30 °C) are acclimated to cold water (<15 °C) or hypoxia, respectively³. Previous studies using the time differential staining technique on goldfish have focused on the redistribution, innervation and proliferation of ICs on the gill in the context of gill remodelling^{4,5}. Katoh and Kaneko¹ developed this novel technique to study the transformation and replacement of branchial ICs in killifish (*Fundulus heteroclitus*) transferred from sea water (SW) to fresh water (FW). In this study, the focus is on the proliferation and innervation of ICs in goldfish acclimated to 25 °C.

Using the time differential staining technique it was shown that, in the context of gill remodelling, goldfish maintain a constant number of ICs during hypoxic exposure and subsequent normoxic recovery, however, the percentage of innervated cells decreased throughout the normoxic recovery period⁵. It was proposed more than 70 years ago that ionic uptake mechanisms in fish are under neural control¹². The teleost gill is innervated by the facial (VII), glossopharyngeal (IX) and vagus (X) nerves also referred to as the "branchial nerves"^{13,14}. Studies by Jonz and Nurse (2003) on zebrafish (*Danio rerio*) gill innervation showed that the origin of innervation is extrinsic (cell body of nerve fiber is extrinsic to the gill) as well as intrinsic (cell body of nerve fiber is intrinsic to the gill). The same authors also demonstrated that branchial ICs are extrinsically innervated⁷.

In this study, the time differential staining technique coupled with full bilateral gill denervation was used to investigate the proliferation of ICs lacking extrinsic innervation in goldfish. Full bilateral gill denervation refers to severing cranial nerves IX and X. These two approaches are feasible in goldfish because their relatively large size (from 30 - 200 g) simplifies the delicate surgical procedures, and ionocytes are readily identified using standard immuno-histochemical techniques. In the present study, ICs were visualized using a vital mitochondrion-specific

dye (e.g., MitoTracker Red) or a primary antibody against the α -subunit of the Na^+/K^+ -ATPase (α -5; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City IA). This protocol provides a straightforward method of visualizing and analysing the redistribution and proliferation of ICs on the fish gill.

Protocol

Both protocols conformed to the guidelines of the Canadian Council of Animal Care (CCAC) and were carried out with the approval of the University of Ottawa Animal Care Committee (Protocol BL-226).

1. Time Differential Staining Technique: Mitochondrion-rich Dye Bath

1. Prepare 1 mM MitoTracker Red stock solution by dissolving 50 μg in 94.0 μl of dimethyl sulfoxide (100% DMSO). Keep stock solution in the dark at -20°C when not in use. Avoid freeze/thaw cycles.
2. Prepare dark boxes (3 - 6 boxes) with a maximum volume of 600 ml. Fill the boxes with 400 ml of system water (water the fish are normally held in) and place an air stone in each box to provide a source of O_2 . Obtain goldfish (30 - 40 g) and place them in the boxes with 400 ml of water and an air stone. After 30 min, add the viable mitochondrion-rich dye to yield final concentrations of 0.1 μM and 0.01% DMSO. Bathe the fish for 4 hr.
 1. If these are control fish (*i.e.*, no denervation), turn on water flow to the boxes, allow the dye to flush out and recover the fish for the period of time allotted in the protocol. Fish are typically recovered for 3 - 5 days.
3. After the recovery period proceed to **Section 3: Time Differential Staining Technique: Immunohistochemistry**. If these fish are to be denervated proceed to **Section 2: Full Bilateral Denervation Procedure**.

2. Full Bilateral Denervation Procedure

1. Obtain 1 pair of student Vannas spring scissors (curved), 1 pair of standard pattern forceps-straight, 1 pair of standard pattern forceps-curved, 2 pairs of No. 5 forceps, 1 pair of tissue retractors, small cotton balls (1 - 2 mm in diameter), and cotton swabs (Q Tips or equivalent).
2. Prepare the anaesthetic water bath. First, dissolve 10 g of benzocaine to a final volume of 100 ml of 99% ethanol to prepare a stock solution. To prepare the anaesthetic water bath, dissolve 15 ml of the stock benzocaine solution into 30 L of aerated system water at the required temperature. Aerate the water by placing an air-stone connected to an air pump or a central air-line into the water tank.
 1. Place fish into the anaesthetic water bath (**Figure 1A**).
 2. Once breathing has ceased, place the fish on a surgery table and intubate it as shown in **Figure 1B**. Do this by inserting a tube in its buccal cavity to irrigate the gills with aerated anaesthetic solution. Irrigation of the gills ensures that the fish is supplied with a sufficient amount of O_2 and anaesthetic during the denervation procedure. Position the fish so that the head is tilted slightly downward. This allows for better access to the area behind the fourth gill arch.
3. Gently lift the operculum with the straight standard pattern forceps and place the tissue retractors between the operculum and the inside of the head. Carefully open the tissue retractors to keep the operculum away from the head and keep the gills exposed.
4. Make sure that the anaesthetic solution is irrigating the gills during this procedure. Rest the retractor handles next to the head which provides access to all four gill arches.
5. Place the curved standard pattern forceps between the fourth gill arch and the back of the head and gently open them to create tension in the ligament attaching the fourth gill arch to the head. With a pair of No. 5 forceps create a small opening (2 - 3 mm) by piercing the epithelium connecting the dorsal end of the gill arches to the opercular cavity. Be careful not to go in too deep because there is the risk of damaging a major blood vessel.
6. With a small cotton ball held with No. 5 forceps slowly and carefully expand the incision to expose the IX (glossopharyngeal) and X (vagus) nerves. Free the nerves from any connective tissue by using the No. 5 forceps, again taking care not to damage blood vessels.
NOTE: The branchial IX and X nerves of the goldfish rest deep behind the fourth gill arch and are in proximity to major blood vessels feeding into the gill arches.
7. Once nerves have been identified use the curved spring scissors to carefully cut the nerves while holding the incision open with the curved standard shape forceps. After severing the nerves, gently retract the curved forceps. There is no need to close the incision with sutures because the epithelium is very thin and the incision usually closes on its own within 24 - 48 hr. Remove tissue retractor.
8. Repeat the same procedure on the other side of the head.
9. Switch the irrigation of the gills from anaesthetic to fresh aerated water to recover the fish from anaesthesia. Once opercular movements have resumed move the fish into experimental tanks to recuperate for at least 24 hr.
10. Perform a "sham" procedure on a separate set of fish. The "sham" procedure involves piercing the epithelium behind the fourth gill arch without severing the nerves.

3. Time Differential Staining Technique: Immunohistochemistry

1. First, prepare 4% paraformaldehyde (PFA) in 1x phosphate buffered saline (PBS; 4 g PFA in 96 ml of PBS). PFA does not dissolve easily in PBS at RT. Heat solution in a water bath to dissolve PFA. Perform this in a fume hood. Once PFA is in solution let cool before use. Store at 4°C for up to 2 weeks.
2. Before euthanizing the fish and extracting the gill tissue, place 3 - 4 ml of 4% PFA into a scintillation vial for a total of 8 scintillation vials (1 vial per gill arch). In addition, take a small weigh boat and fill it with 1x PBS. This will be used to wash the tissue after it has been excised. Keep all solutions on ice.
3. After the viable mitochondrion-rich dye exposure experiment has finished, euthanize the goldfish by placing it in a water bath with an overdose of benzocaine.

4. Use blunt forceps to lift the operculum on one side of the head and curved scissors to sever each end of the branchial gill basket. Carefully pick up the gills by the rakers using blunt forceps and lift them out of the opercular cavity. Immediately wash the gills in ice cold 1x PBS to remove excess benzocaine and blood.
5. Place the excised gills in separate vials (a vial for each gill arch) filled with 4% PFA and fix O/N at 4 °C.
6. After fixation, wash the excess PFA in 1X PBS and place the tissue in a 2 ml bullet tube filled with 1.5 ml of 1% Triton-X on a shaker for 6 hr at RT or O/N at 4 °C. This step permeabilizes the tissue. If the whole gill is too big to place into a 2 ml tube then cut the tissue into sections small enough to fit the tube taking care not to damage the filaments.
 1. Prepare the primary antibody dilutions by mixing 4 µl of the stock solution of each primary antibody (total of 8 µl) into 992 µl of 1x PBS. Make sure that the NKA (labels NKA-rich cells) and zn-12 (labels neurons) primary antibodies have been raised in the same host. This is important if the researchers decide to identify specific IC subtypes at the same time for which they will have to use primary and secondary antibodies raised in a different host species.
 2. Remove the Triton-X solution and without washing the tissue add a 1:250 dilution (dilute in 1x PBS) of a NKA monoclonal antibody (α-5) to detect NKA-rich cells and a zebrafish specific neuronal antibody (zn-12) to detect nerve fibers (primary antibodies) and incubate on a shaker for 6 hr at RT or O/N at 4 °C.
7. Wash the primary antibody 3 times for 3 min each using 1x PBS. To do this, remove the primary antibody solution from the tube by suctioning it out using a pipette.
8. Prepare the secondary antibody at a 1:200 dilution by mixing 5 µl of stock secondary antibody into 995 µl of 1x PBS. Apply secondary antibody (Alexa Fluor 488) and incubate for 6 hr at RT or O/N at 4 °C on a shaker.

NOTE: MitoTracker Red is excited at a ~594 nm wavelength and will fluoresce red. A secondary antibody that is excited at a ~488 nm wavelength and fluoresces green must be used to label NKA and zn-12 primary antibodies.
9. Remove excess secondary antibody by washing the tissue 3 times for 5 min each (as described in step 3.7).

4. Imaging

1. After the washes, mount the tissue on a concave slide for whole mount confocal imaging of cells and nerve fibers. To mount the tissue, first place it in a drop (200 µl) of 1x PBS on a flat microscope slide. This ensures that the tissue does not desiccate.
2. Separate the gill hemibranchs with the curved micro-scissors. Place a drop of 1x PBS and a drop of mounting media into a concave slide.
3. Place the separated hemibranchs into the concave slide with the leading edge of the filament facing upward and cover with a cover slip. Dab the edges of the cover slip with nail polish in order to prevent the cover slip from moving around and displacing the tissue. Allow the tissue to settle to the bottom of the concave slide for 10 - 15 min before imaging.
4. For each gill arch, select six gill filaments at random for imaging, producing six images per gill arch. Use conventional confocal microscopy to image the tissue by taking 1 - 3 µm optical slices.

NOTE: Any pre-existing cells will be labelled with MitoTracker Red and positive for NKA will appear yellow only. Cells that appear red only are pre-existing ICs that do not contain NKA. Any newly proliferated cell will only be positive for NKA and will appear green only. Nerve fibers will also appear green.

5. Image Analysis for Ionocyte Quantification

1. For each gill filament that has been imaged, quantify the ICs and associated innervation by scrolling through the sections of the Z stack and counting the number of lamellar and filamental ICs present and whether or not they are newly differentiated, pre-existing, and/or innervated.
2. Quantify the ICs per filament or per area (mm²) of filament. Do this by using the drawing tools associated with the software used to acquire the images to outline the lamellae of the filament encompassing the area of the filament in which the ICs were quantified. Most confocal imaging software programs have the option to calculate the area of an outlined region on the image. Use the option in the imaging software which allows you to do this to acquire the area.
3. Divide the counted ICs by the area calculated by the software to obtain a measure of ICs per unit area (e.g., per mm²).

Representative Results

Figure 1 illustrates the surgery table set up (**Figure 1A**), the placement of the fish during surgery (**Figure 1B**) and the three most important steps for the time differential staining technique (**Figure 1C**). In Step 1, the fish is kept for 30 min in a well aerated water bath at 25 °C in the dark. During the 30 min period, the researcher can prepare the mitochondrion-rich dye aliquot in DMSO which is added to the water during Step 2 (**Figure 1C**). The incubation period in Step 2 allows for uptake of the mitochondrion-rich dye from the water into the mitochondrion-rich cells (*i.e.*, ICs). The fish can then either undergo the full bilateral denervation procedure or a sham procedure in which the fish is anaesthetized and the opercula manipulated but the nerves remain intact. The set up in Step 3 represents a recovery chamber provided with flowing water for a fish that has either undergone full bilateral denervation or a "sham" procedure.

After the recovery period, the fish was euthanized and the gills were excised and fixed for immunohistochemistry. The overall distribution and innervation of ICs on the gill filament of a fish that had undergone a "sham" procedure is depicted in **Figure 2**. The ICs are present on the filamental epithelia as well as at the base of the interlamellar regions. **Figure 2A** shows pre-existing ICs labelled with mitochondrion-rich dye (*i.e.*, these ICs existed before the denervation/sham procedures were performed). **Figure 2B** shows nerve fibres innervating the pre-existing and newly formed ICs (identified by NKA immunoreactivity) of the filamental and lamellar epithelia. Finally, merging of the two images (**Figure 2C**) clearly reveals the pre-existing ICs (appear yellow) and the new ICs (appear green). **Figure 2D** is a representative graph of IC quantification for the filament depicted in **Figure 2A-C**. On this specific filament, there appear to be a larger number of newly proliferated ICs per mm² than pre-existing ICs (N = 1). To remove the source of extrinsic branchial innervation, the IX and X cranial nerves (extrinsic innervation) were severed. **Figure 3A** shows the dorsal region of the opercular cavity after the gill filaments and the epithelia covering the nerves were removed to expose the IX and X cranial nerves (indicated with Roman numerals) which span from two major nerve trunks to innervate all four arches (**Figure 3A**); the gill arches are numbered 1 through 4. **Figure 3B-E** illustrate selective denervation of the first gill arch. The first gill arch is innervated by

both the IX and X cranial nerves (**Figure 3B**) which can be removed without affecting the innervation to the rest of the gill arches (**Figure 3C-E**). Full bilateral denervation results in gradual loss of extrinsic innervation to the gill filaments (**Figures 4A-C**). Control fish exhibit an obvious nerve bundle which spans the length of the filament (**Figure 4A**). Full bilateral denervation resulted in some loss of extrinsic innervation after 2 days of recovery (**Figure 4B**). Further disappearance of extrinsic innervation was noted after 5 days of recovery from denervation (**Figure 4C**). Any remaining innervation to ICs after 5 days of recovery presumably was derived from nerves with cell bodies within the gill filament (intrinsic innervation; **Figure 4C**).

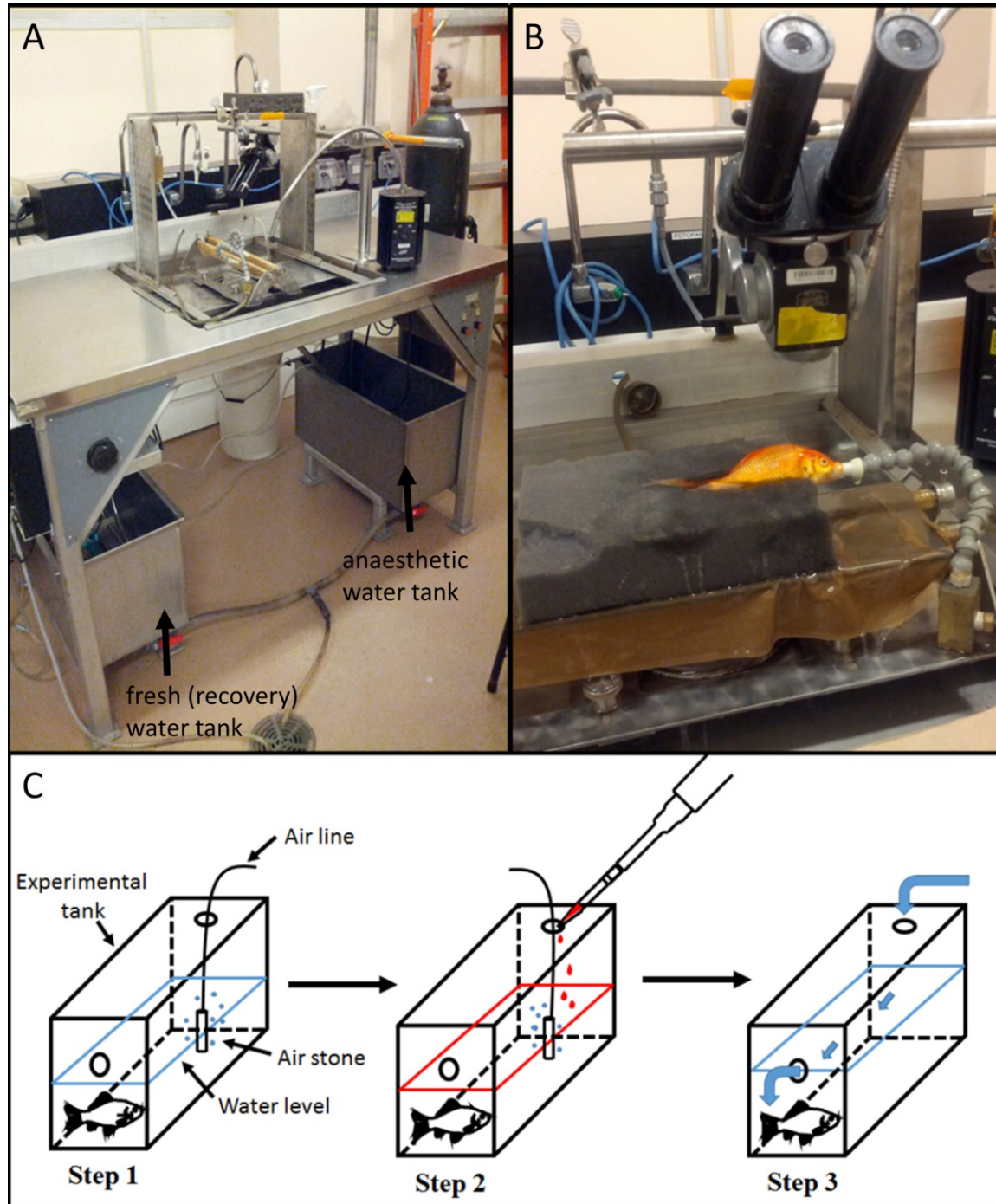


Figure 1. Experimental set up for the denervation procedure and sequence of steps used in the time differential staining technique. (A) Surgery table set up for denervation procedure showing the anaesthetic and recovery tanks. **(B)** Example of the placement of an anaesthetized, intubated fish. **(C)** Key steps used in the time differential staining technique. In Step 1, the fish is placed in a temperature controlled, static aerated water bath for 30 min. The mitochondrion-rich dye is added in Step 2 to a final concentration of 0.1 μM and the fish is allowed to bathe in the solution for a minimum of 4 hr. Water flow is restarted in Step 3 at which point the fish undergoes either a full bilateral denervation of the gill or a sham surgery. [Please click here to view a larger version of this figure.](#)

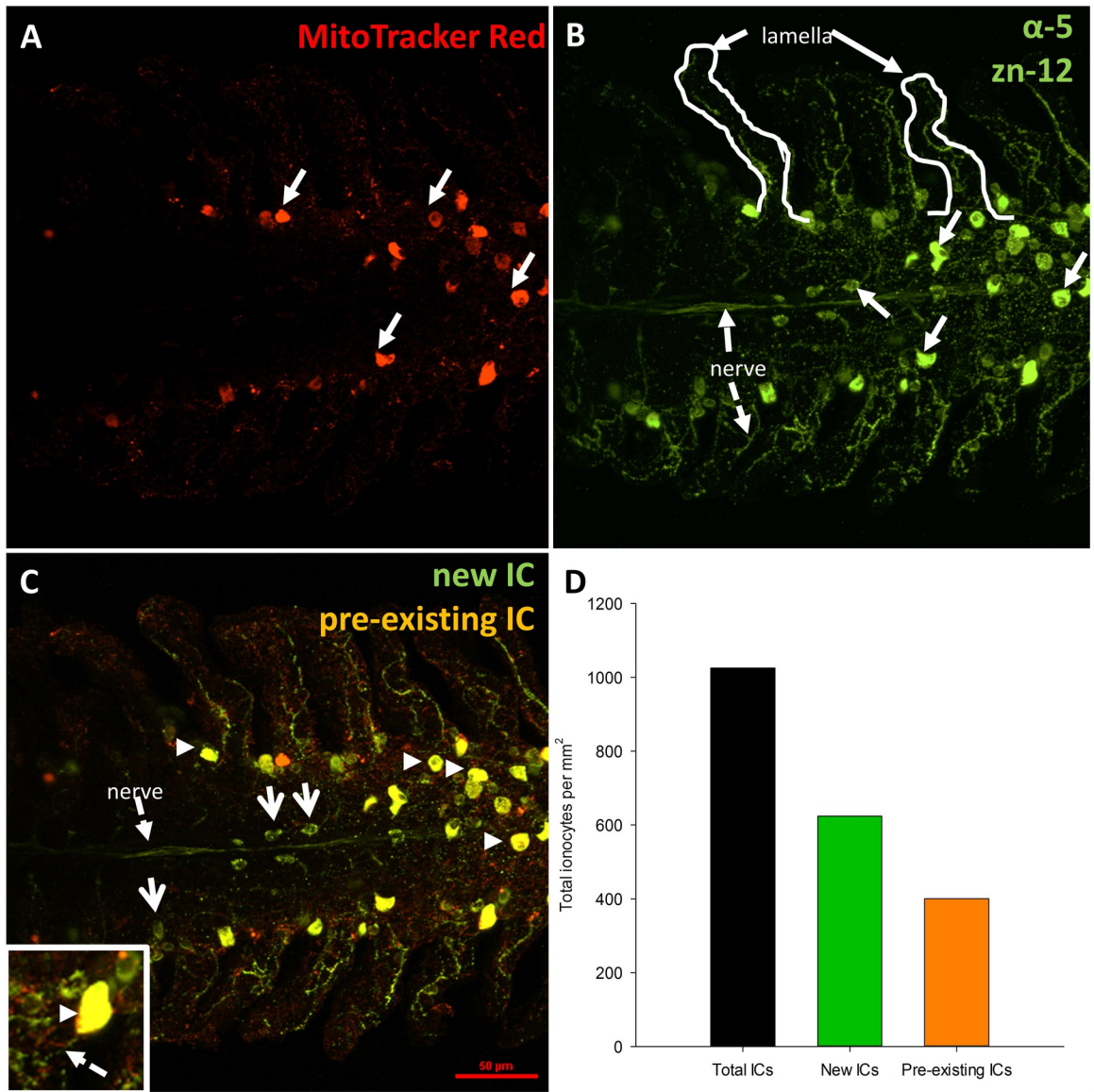


Figure 2. Light micrographs representing the time differential staining technique in a goldfish (acclimated to 25 °C) 2 days after full bilateral denervation. **(A)** The distribution of pre-existing ionocytes (ICs; arrows) on a single gill filament is revealed by mitochondrion-rich dye staining. **(B)** The distribution of ICs (new and pre-existing) and branchial nerves (dashed arrows) is revealed by staining with the α -5 and zn-12 antibodies, respectively. The arrows indicate ICs. **(C)** The overlap of **(A)** and **(B)** distinguishes pre-existing ICs (appear yellow; indicated by arrowheads) from newly proliferated ICs (appear green; indicated by arrows). Insert in **(C)** is a magnification of an innervated pre-existing ionocyte. **(D)** Representative graph of IC quantification for the filament shown in panels **(A-C)**. N = 1. Scale bar in panel **(C)** is 50 μ m and applies to all panels. [Please click here to view a larger version of this figure.](#)

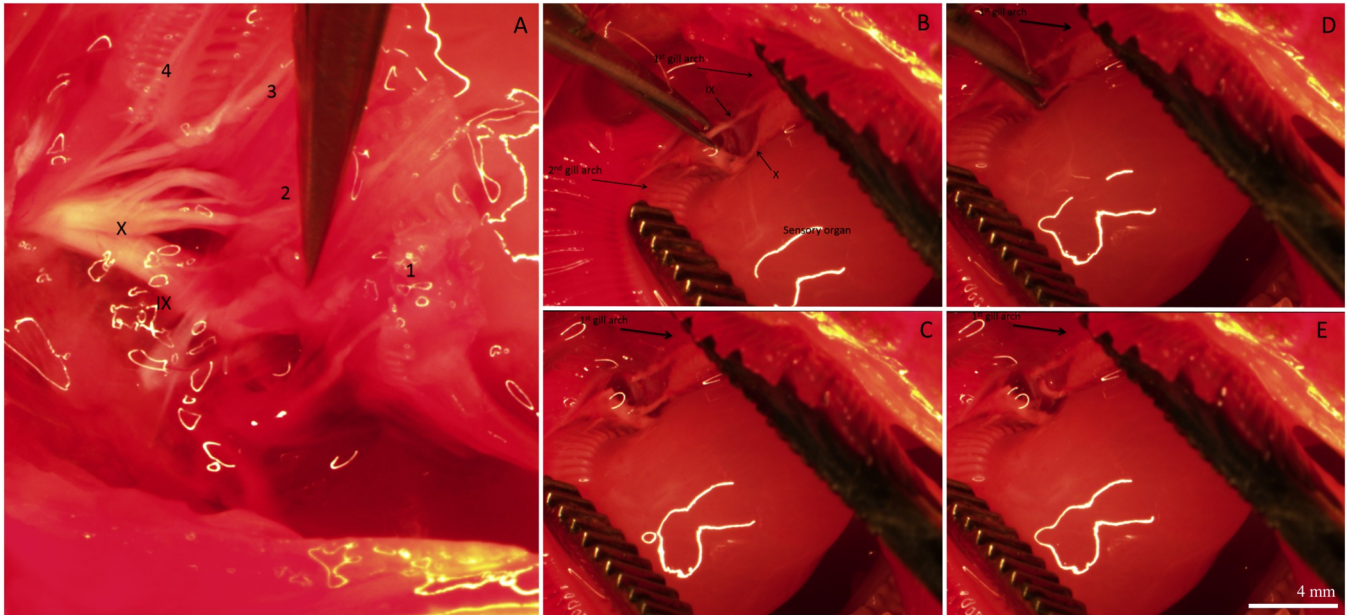


Figure 3. Representative images depicting various stages of the gill innervation. (A) Dorsal view of the buccal cavity showing the IX and X cranial nerves innervating all 4 gill arches. Gill arches are numbered 1-4. The gill filaments and the tissue covering the nerves were removed to better visualize the innervation. (B) The 1st and 2nd gill arches are separated to reveal the sensory organ and the branches of the IX and X cranial nerves innervating the 1st gill arch. (C-E) Sequence of images showing the selective denervation of the 1st gill arch by severing the branches of the IX and X cranial nerves. For a schematic representation of gill innervation of teleost fish, refer to Figure 1 in Milsom *et al.*²⁶ The white lines in the images are water reflection from the microscope lights; they do not define any morphological structure. Scale bar in panel (E) is 4 mm and applies to all panels. [Please click here to view a larger version of this figure.](#)

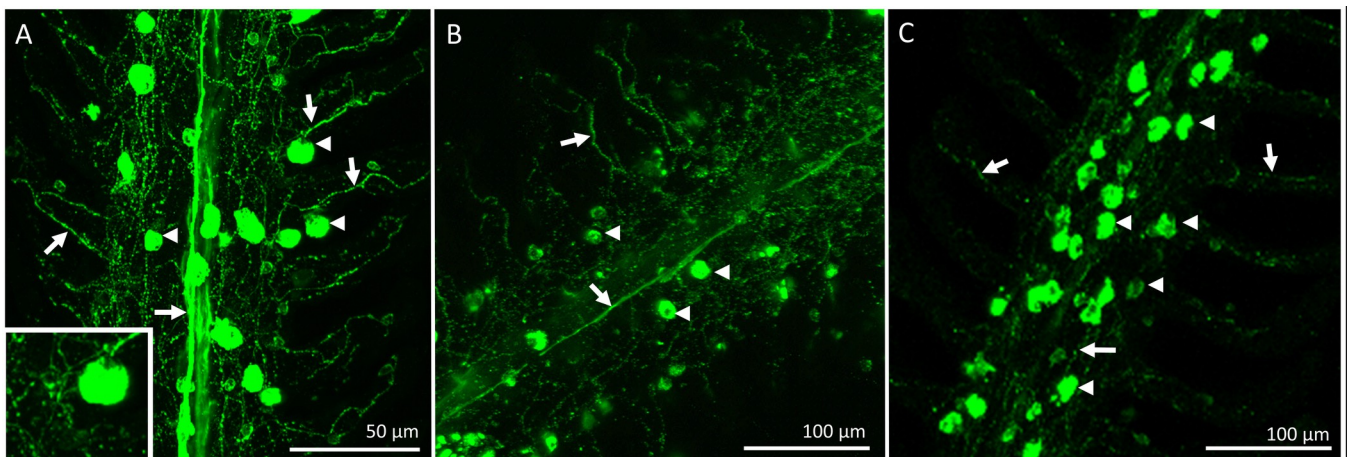


Figure 4. Light micrographs depicting the distribution and innervation of ionocytes on a single filament of the 1st gill arch of a goldfish acclimated to 25 °C. Ionocytes (indicated by arrow heads) were stained with the α -5 antibody and nerves (indicated by arrows) were stained with the zn-12 antibody. (A) Gill filament of a control fish showing a central nerve bundle presumably originating from the IX and X nerves (extrinsic innervation) with extensive lamellar branching. Some of the ionocytes indicated are also innervated (insert). (B) A gill filament 2 days after full bilateral denervation. There was a reduction of the central nerve bundle while the lamellar innervation appeared largely intact. (C) A gill filament 5 days after full bilateral denervation demonstrating that extrinsic innervation was largely absent. Qualitative analysis suggests that full bilateral denervation causes degradation of the extrinsic gill innervation while maintaining nerves with cell bodies within the gill filament (intrinsic innervation) creating a network of nerves through the filament and into the lamellae. [Please click here to view a larger version of this figure.](#)

Discussion

The time differential staining technique can be a useful tool to understand the dynamic regulation of ion uptake and to examine the temporal redistribution of ICs in the gill epithelia. Although a straightforward procedure, there are a number of key points that are crucial to the success of the time differential staining technique. The goldfish must be exposed to the mitochondrion-rich dye for the time allotted in the protocol. Shorter exposures will result in poor uptake of the dye by the mitochondrion-rich cells (*i.e.*, ionocytes). During fixation, the gill tissue must be excised quickly and kept in the dark to avoid photo bleaching. The tissue must be processed for imaging within 2 weeks of fixation. During the bilateral

nerve sectioning procedure ensure that the fish is well anaesthetized; the nerves and blood vessels are clearly identified; and the fish resumes full opercular function before it is moved to a recovery tank.

The FW environment presents fish with the dual challenge of balancing passive ion losses and osmotic water gain¹⁴. The balancing of passive ion losses occurs via the active uptake of salt across the ICs which are localized to the filamental and lamellar epithelia where they can make direct contact with the external environment^{2,4,8,9,16}. However, the location of the ICs on the gill is not static. Over the past three decades a number of studies have shown that several FW fish species, when faced with an ionic and/or temperature challenge, redistribute branchial ICs from the filament or base of the lamella to the more distal regions of the lamella^{1,2,4,5,17-21}. Such redistribution may increase the thickness of the lamellae which can compromise gas transfer (O₂, CO₂) across the gill epithelia²². Researchers have used the time differential staining technique described in this manuscript to track the relocation and emergence of new ICs on the gill epithelia under these different experimental conditions (**Figure 1C**)^{1,4,5}.

The gills, and presumably the branchial ICs, are innervated by the IX and X cranial nerves^{7,23-25}. These nerves carry both efferent and afferent inputs to and from the gill, respectively. They are located at the dorsal side of the buccal cavity behind the 4th gill arch. The accessibility of the nerves and the ease with which the bilateral denervation procedure can be performed is species specific. In trout, for example, the pointed and flattened anatomy of the head allows for the nerves to lie in a single plane behind the 4th gill arch under a thin layer of tissue. This makes the nerves visible and easily accessible to the researcher to perform the denervation procedure. In contrast, goldfish have a shorter snout and a rounder head. The IX and X cranial nerves of goldfish lie deeper into the dorsal side of the cavity after the 4th gill arch occupying different planes. This orientation limits the ease of access to the nerves and requires a more careful approach to identify and sever the appropriate nerves. The aim of the denervation procedure is to remove sensory afferent and efferent input to and from the gill, respectively. Denervation of the gill arches can also be coupled with ion flux experiments using radioisotopes (e.g., ²²Na). These techniques can be used in tandem to study the contribution of nervous input on ion movement across the gill epithelia. Another limitation to the denervation procedure is the inability to distinguish between sensory and motor neurons, thus when severing the nerve bundle it is possible that both types of innervation are being removed. Severing any motor neurons may affect the gill and opercular movement of the fish. Thus when performing gill denervation experiments it is important to also monitor ventilation after the fish has recovered from the procedure to ensure that there is sufficient gill movement for both gas and ion exchange.

The protocols described in this manuscript use adult animals kept on a 12:12 light:dark cycle and fed commercial food pellets. These methods can be modified in several ways. First, the recovery period after mitochondrion-rich dye exposure can be adjusted to the requirements of the researcher's protocol (e.g., 1, 3, 5, or 14 days). The longest period of recovery after mitochondrion-rich dye exposure in the lab has been 14 days^{4,5}. There wasn't a significant decrease in the intensity of the fluorescence of the mitochondrion-rich dye after 14 days of recovery. Second, the use of the α -5 primary antibody is limited to only identifying NKA-rich cells and does not distinguish between the different subtypes of branchial ICs. Fortunately, in goldfish it was established that the majority of ICs are both mitochondrion-rich (label with MitoTracker) and NKA-rich (label with α -5) cells which may not be the case in all fish species^{4,27}. Future experiments can focus on following the temporal redistribution of specific IC subtypes by using antibodies directed specifically against a variety of channels, pumps and exchangers (e.g., NHE, H⁺ pump). Previous studies found that the majority of ionocytes stained with mitochondrion-rich dye exhibit NKA immunoreactivity⁴. Innervation of the ICs can be detected by using a primary antibody against zebrafish-derived neuron-specific antigen (zn-12). In this study, α -5 and zn-12 primary antibodies were detected by using the same secondary antibody (Alexa Fluor 488) for both. This limitation is due to both primary antibodies being raised in a mouse host and is overcome by the fact that the NKA-rich cells and neurons can be distinguished morphologically even though they fluoresce the same colour. Sequential staining with different secondary antibodies (e.g., first α -5 with Alexa Fluor 488 then zn-12 with Alexa Fluor 594) can also be used to eliminate the problem of having both markers fluorescing the same color. Lastly, the full bilateral nerve sectioning protocol can be modified to target nerves to specific gill arches. For example, selective nerve sectioning can be performed on the first gill arch by gently separating the first and second gill arches to expose the nerves leading to the first gill arch at the dorsal end of the gill basket (**Figure 2B-E**). The time differential technique can also be applied to study the distribution of ionocytes in larval zebrafish fish as they develop and ion transport transitions from the skin to the gill.

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Katoh, F., Kaneko, T. Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established 'time-differential double fluorescent staining' technique. *J. Exp. Biol.* **206**, (22), 4113-4123 (2003).
2. Perry, S. F. Relationships between branchial chloride cells and gas transfer in freshwater fish. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **119**, (1), 9-16 (1998).
3. Sollid, J., Weber, R. E., Nilsson, G. E. Temperature alters the respiratory surface area of crucian carp *Carassius carassius* and goldfish *Carassius auratus*. *J. Exp. Biol.* **208**, (6), 1109-1116 (2005).
4. Mitrovic, D., Perry, S. F. The effects of thermally induced gill remodeling on ionocyte distribution and branchial chloride fluxes in goldfish (*Carassius auratus*). *J. Exp. Biol.* **212**, (6), 843-852 (2009).

5. Tzaneva, V., Vadeboncoeur, C., Ting, J., Perry, S. F. Effects of hypoxia-induced gill remodelling on the innervation and distribution of ionocytes in the gill of goldfish, *Carassius auratus*. *J. Comp. Neurol.* **522**, (1), 118-130 (2014).
6. Greco, A. M., Fenwick, J. C., Perry, S. F. The effects of soft-water acclimation on gill structure in the rainbow trout *Oncorhynchus mykiss*. *Cell Tissue Res.* **285**, (1), 75-82 (1996).
7. Jonz, M. G., Nurse, C. A. Epithelial mitochondria-rich cells and associated innervation in adult and developing zebrafish. *J. Comp. Neurol.* **497**, (5), 817-832 (2006).
8. Laurent, P., Hebibi, N. Gill morphometry and fish osmoregulation. *Can. J. Zool.* **67**, (12), 3055-3063 (1989).
9. Perry, S. F., Laurent, P. Adaptational responses of rainbow trout to lowered external NaCl concentration: contribution of the branchial chloride cell. *J. Exp. Biol.* **147**, 147-168 (1989).
10. Evans, D. H., Piermarini, P. M., Choe, K. P. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, (1), 97-177 (2005).
11. Hwang, P. P., Lee, T. H., Lin, L. Y. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, (1), R28-R47 (2011).
12. Krogh, A. The Active Uptake of Ions into Cells and Organisms. *Proc. Natl. Acad. Sci. U. S. A.* **25**, (6), 275-277 (1939).
13. Nilsson, G. E. Innervation and pharmacology of the gills. *Fish Physiology*. Randall, D. J., Hoar, W. S. 185-272 Academic Press Orlando, FL (1984).
14. Sundin, L., Nilsson, S. Branchial innervation. *J. Exp. Zool.* **293**, (3), 232-248 (2002).
15. Dymowska, A. K., Hwang, P. P., Goss, G. G. Structure and function of ionocytes in the freshwater fish gill. *Respir. Physiol. Neurobiol.* **184**, (3), 282-292 (2012).
16. Witters, H., Berckmans, P., Vangenechten, C. Immunolocalization of Na⁺/K⁺-ATPase in the gill epithelium of rainbow trout, *Oncorhynchus mykiss*. *Cell Tissue Res.* **283**, (3), 461-468 (1996).
17. Bindon, S., Fenwick, J. C., Perry, S. F. Branchial chloride cell proliferation in the rainbow trout, *Oncorhynchus mykiss*: implications for gas transfer. *Can. J. Zool.* **72**, (8), 1395-1402 (1994).
18. Bradshaw, J. C., Kumai, Y., Perry, S. F. The effects of gill remodeling on transepithelial sodium fluxes and the distribution of presumptive sodium-transporting ionocytes in goldfish (*Carassius auratus*). *J. Comp. Physiol. B.* **182**, (3), 351-366 (2012).
19. Chou, M. Y., *et al.* Effects of hypothermia on gene expression in zebrafish gills: upregulation in differentiation and function of ionocytes as compensatory responses. *J. Exp. Biol.* **211**, (19), 3077-3084 (2008).
20. Kaneko, T., Katoh, F. Functional morphology of chloride cells in killifish *Fundulus heteroclitus*, a euryhaline teleost with seawater preference. *Fisheries Science.* **70**, (5), 723-733 (2004).
21. Ouattara, N., *et al.* Changes in gill ionocyte morphology and function following transfer from fresh to hypersaline waters in the tilapia *Sarotherodon melanotheron*. *Aquaculture.* **290**, (1-2), 155-164 (2009).
22. Bindon, S., Gilmour, K., Fenwick, J., Perry, S. The effects of branchial chloride cell proliferation on respiratory function in the rainbow trout, *Oncorhynchus mykiss*. *J. Exp. Biol.* **197**, (1), 47-63 (1994).
23. Dunel-Erb, S., Bailly, Y., Laurent, P. Pattern of gill innervation in two teleosts, the perch and the trout. *Can. J. Zool.* **71**, 18-25 (1993).
24. Jonz, M. G., Nurse, C. A. New developments on gill innervation: insights from a model vertebrate. *J. Exp. Biol.* **211**, (15), 2371-2378 (2008).
25. Jonz, M. G., Zaccone, G. Nervous control of the gills. *Acta Histochem.* **111**, (3), 207-216 (2009).
26. Milsom, W. K., Reid, S. G., Rantin, F. T., Sundin, L. Extrabranchial chemoreceptors involved in respiratory reflexes in the neotropical fish, *Colossoma macropomum* (the tambaqui). *J. Exp. Biol.* **205**, (12), 1765-1774 (2002).
27. Hwang, P., Lee, T. New insights into fish ion regulation and mitochondrion-rich cells. *Comp Biochem Physiol A Mol Integr Physiol.* **148**, (3), 479-497 (2007).