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In situ hybridization in zebrafish larvae and juveniles during mesonephros development

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TITLE:

In Situ Hybridization in Zebrafish Larvae and Juveniles During Mesonephros Development

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KEYWORDS:

zebrafish, kidney, pronephros, mesonephros, lhx1a, larva, juvenile

SUMMARY:

The zebrafish is an important model for understanding kidney development. Here, an *in situ* hybridization protocol is optimized to detect gene expression in zebrafish larvae and juveniles during mesonephros development.

ABSTRACT:

The zebrafish forms two kidney structures in its lifetime. The pronephros (embryonic kidney) forms during embryonic development and begins to function at 2 days post fertilization. Consisting of only two nephrons, the pronephros serves as the sole kidney during larval life until more renal function is required due to the increasing body mass. To cope with this higher demand, the mesonephros (adult kidney) begins to form during metamorphosis. The new primary nephrons fuse to the pronephros and form connected lumens. Then, secondary nephrons fuse to primary ones (and so on) to create a branching network in the mesonephros. The vast majority of research is focused on the pronephros due to the ease of using embryos. Thus, there is a need to develop techniques to study older and larger larvae and juvenile fish to better understand mesonephros development. Here, an *in situ* hybridization protocol for gene expression analysis is optimized for probe penetration, washing of probes and antibodies, and bleaching of pigments to better visualize the mesonephros. The *Tg(lhx1a-EGFP)* transgenic line is used to label progenitor cells and the distal tubules of nascent nephrons. This protocol fills a gap in mesonephros research. It is a crucial model for understanding how new kidney tissues form and integrate with existing nephrons and provide insights into regenerative therapies.

INTRODUCTION:

The zebrafish embryo is an important model for studying tissue development due to its small

size, transparency, available tools, and survival without feeding for up to five days[1,2]. It has greatly contributed to the understanding of kidney development and the conservation between zebrafish and mammals[3–5]. The kidney plays an essential role in maintaining fluid homeostasis, filtering the blood, and excreting waste[6]. The nephron, the functional unit of the kidney, comprises a blood filter connected to a long tubule. In zebrafish, two kidney structures form throughout its life. The pronephros (the temporary embryonic kidney) forms first during early development. It consists of two nephrons running along the anterior-posterior axis and becomes functional at around 2 days post fertilization (dpf). The utility of the pronephros lies in its simplicity, having just two nephrons that are mostly linear and easy to visualize (although the proximal convoluted tubule begins to coil at three dpf)[3]. This has facilitated not only studies of its early development from the intermediate mesoderm, but also the segmentation pattern and tubule repair[7,8].

The usefulness of zebrafish becomes limited after five dpf, when the yolk is diminished and the larvae rely on feeding in the aquatic system[9]. At around 2 weeks old, the larvae undergo metamorphosis into juveniles, where new tissues form and old tissues are lost and/or reorganized[9]. This is also when the mesonephros (the permanent adult kidney) forms[10–12]. The first adult nephron forms near the sixth somite, and fuses with the distal early tubule of the pronephros. Additional nephrons are added posterior to this position initially, but also toward the anterior later on. The primary nephrons in this first wave fuse to the tubules of the pronephros and share a common lumen to deposit their waste. Secondary nephrons form in the next wave and fuse to primary nephrons. This reiterative process creates a mesonephros that is branched, somewhat akin to the mammalian kidney. Presumably, the pronephros eventually loses its tubule identity and becomes two major collecting ducts where all nephrons drain their waste[13].

Prior to the formation of the first adult nephron, single progenitor cells begin to appear in ~4 mm (total length) larvae (~10 dpf). These cells, which are marked in the *Tg(lhx1a-EGFP)* transgenic line, adhere to the pronephros and seem to migrate to future sites of nephrogenesis. The single cells coalesce into clusters, which differentiate into new nephrons[12]. It is unclear where these cells reside or what genes they express before the onset of mesonephrogenesis.

Understanding mesonephros development provides insights into the mammalian kidney in ways that the pronephros cannot. These include the formation of nephrogenic aggregates from single progenitor cells, functional integration of new nephrons with existing ones, and branching morphogenesis. However, there are limitations to studying postembryonic development. The larvae are less transparent due to their larger size and having pigmentation. The developmental timeline is not synchronous among individual animals and is highly dependent on environmental factors, such as feeding and crowding[9,14]. Although knockdown reagents exist, they are less effective in larvae compared to embryos[15]. In this protocol, an *in situ* hybridization method to determine gene expression during zebrafish mesonephros development is described. Several steps are optimized to increase visualization of the mesonephros and penetration and washing of the probe and antibody. The *Tg(lhx1a-EGFP)* transgenic line is used along with a probe for EGFP to label single progenitor cells, nephrogenic aggregates, and the distal tubules of nascent

nephrons. This method will provide a deeper understanding of mesonephros development and insight into regenerative therapies.

PROTOCOL:

The use of zebrafish larvae and juveniles is approved by the IUP IACUC (protocol #02-1920, #08-1920). Details of the solution content are listed in the **Table of Materials**.

1. Raising larvae

NOTE: It will take up to 21 days or more to raise larvae and juveniles to the stage of interest.

1.1. Set up adult zebrafish to mate by adding 1 male and 1 female fish in a mating tank in the late afternoon after their last meal.

NOTE: Not all fish pairs will mate. Start by setting up 20 pairs to determine the mating rate and adjust accordingly in future experiments.

1.2. The next day after the fish have finished mating (around 1 pm), collect the embryos with E3 medium in Petri plates.

1.2.1. Ensure that there are no more than 30 embryos per plate.

1.2.2. Remove debris (such as feces) from the Petri plates and add 20 mL of E3 medium.

1.2.3. Incubate at 28.5 °C for 1 day.

1.3. Put the adult zebrafish back in the aquatic system.

1.4. At 1 dpf, replace the E3 medium with fresh E3.

1.4.1. Incubate at 28.5 °C for 4 more days until 5 dpf.

1.5. Put a 400 µm screen in a 2.8 L tank and fill it with 2 cm of system water.

1.5.1. Add the 5 dpf larvae from 1 plate (up to 30 total larvae).

1.5.2. Put the tank in the aquatic system, but do not start the water flow.

1.6. Feed the larvae 4 mL of powder food twice a day until 14 dpf.

1.7. At 6 dpf, start the water flow at 1 drip per second.

NOTE: Check the waterflow daily and adjust accordingly.

1.8. At 8 dpf, increase the water flow to a slow, steady stream.

1.9. At 14 dpf, feed live brine shrimp in addition to the powder food.

2. Day 1–2: Fixing larvae

2.1. Remove a tank of larvae at the desired timepoint.

2.2. Fill a Petri plate with 20 mL of system water.

2.3. Use a fine mesh net to gently scoop the larvae and bring them to the water surface.

2.3.1. Cut off the tip of a transfer pipette to give a wider opening and transfer the larvae to the Petri plate. The larger pipette mouth allows for the transfer of several small larvae or a few larger ones at once.

2.4. Add 2 mL of tricaine (2%, pH 7 stock) to immobilize the larvae.

2.4.1. After the larvae stop moving, remove most of the water and add 10 mL of tricaine. Wait for 15 min for the larvae to be euthanized.

2.4.2. Optional: For juveniles longer than 8 mm, cut off the heads and tails with a razor blade (after euthanasia) under a dissecting microscope to improve penetration of the fixing solution. Make sure to record the total length of each animal before cutting off the head and tail and isolate each one in its own Petri plate. Refer to step 3 for how to measure the animals.

2.5. Replace the tricaine with 20 mL of fixing solution (4% paraformaldehyde, 1% DMSO). Put the lid back on the Petri plate and rock slowly in a fume hood.

NOTE: It is important to use a rocking platform. A shaking platform with a circular motion will cause the animal axis to be curved. Use only fresh (not premade frozen) fixing solution.

CAUTION: The fixing solution contains paraformaldehyde, which is a probable carcinogen. Use fume hood (or mask) and gloves to measure the powder.

2.6. After 30 min, replace the fixing solution with a fresh fixing solution.

2.6.1. Collect the used fixing solution in a separate waste container for proper disposal.

2.6.2. Transfer the larvae into a 50 mL tube containing 25 mL of fresh fixing solution.

2.6.3. Make sure the cap is tight and rock slowly at 4 °C for 2 days. It is possible to incubate longer for convenience.

3. Day 3: Measuring larvae

3.1. In a fume hood, replace the fixing solution with 20 mL of PBST.

3.2. Transfer the larvae into a Petri plate.

3.3. Put a flat ruler on a dissecting microscope and put the Petri plate on top of the ruler.

3.4. Use an eyelash manipulator to move each larva onto the ruler to measure its total length (from the snout to the tip of the caudal fin) (**Figure 1**).

3.5. Combine several larvae of similar lengths (e.g., 5.0 mm and 5.1 mm) into one 5.5 mL glass vial, up to 10 larvae per vial.

4. Day 3–4: Dehydration

4.1. Replace the PBST with 4 mL of 100% methanol. Rock the vial for 5 min at room temperature.

4.2. Replace the methanol with fresh methanol and rock for 5 min. Repeat one more time.

4.3. Store the vial at -20 °C for 2 days. It is possible to incubate longer for convenience.

5. Day 5: Rehydration

5.1. Warm the vial to room temperature.

5.2. Replace the methanol with 4 mL of 75% methanol/25% PBST. Rock for 5 min at room temperature.

5.3. Replace the solution in step 5.2 with 4 mL of 50% methanol/50% PBST and rock for 5 min.

5.4. Replace the solution in step 5.3 with 4 mL of 25% methanol/75% PBST and rock for 5 min.

5.5. Replace the solution in step 5.4 with 4 mL PBST and rock for 10 min.

5.6. Replace the PBST with 4 mL of fresh PBST and rock for 10 min. Repeat one more time.

6. Day 5: Proteinase K digest

6.1. Replace the PBST with 2 mL of the proteinase K solution (20 µg/mL, 1% DMSO final concentration in PBST) and rock at room temperature for 30 min.

NOTE: Larvae longer than 6 mm will need a longer incubation time and/or a higher proteinase K

concentration. The exact time and concentration will need to be determined empirically. Start with a 10 min increase in incubation time for every 0.5 mm longer than 6 mm.

6.2. Replace the proteinase K solution with 4 mL of PBST and rock for 10 min.

6.3. Replace the PBST with 4 mL of fresh PBST and rock for 10 min. Repeat one more time.

6.4. Replace the PBST with 4 mL of fresh fixing solution and rock for 1 h. It is possible to incubate longer at 4 °C for convenience.

7. Day 5: Bleaching

7.1. Replace the fixing solution with 4 mL of PBST and rock at room temperature for 10 min.

7.2. Replace the PBST with 4 mL of fresh PBST and rock for 10 min. Repeat one more time.

7.3. Transfer the larvae to a 6-well plate (up to 10 larvae per well).

7.4. Replace the PBST with 3 mL of fresh bleaching solution.

7.5. Rock at room temperature and monitor the pigmentation under a dissecting microscope every 5 min. Look for the disappearance of pigmentation along the mesonephros (dorsal to the swim bladder and gut).

NOTE: For larvae up to 6 mm long, it will take up to 20 min to bleach the pigmentation surrounding the mesonephros. Do not bleach longer than necessary to preserve the integrity of the larvae.

7.6. Transfer the larvae back into a glass vial.

7.7. Replace the bleaching solution with 4 mL of PBST. Rock for 10 min.

7.8. Replace the PBST with 4 mL of fresh PBST and rock for 10 min. Repeat one more time.

7.9. Replace the PBST with 4 mL of fresh fixing solution and rock for 1 h. It is possible to incubate longer at 4 °C for convenience.

8. Day 5: Prehybridization

NOTE: For steps done at 70 °C, it is important to work quickly to minimize the vial cooling down.

8.1. Replace the fixing solution with 4 mL of PBST and rock at room temperature for 10 min.

8.2. Replace the PBST with 4 mL of fresh PBST and rock for 10 min. Repeat one more time.

8.3. Replace the PBST with 4 mL of the Hyb- solution, and rock at room temperature for 10 min.

CAUTION: The Hyb-, Hyb+, and probe solutions contain formamide, which can irritate the skin. Use gloves to handle these solutions.

8.4. Replace the Hyb- with 4 mL of fresh Hyb- and rock for 10 min. Repeat one more time.

8.4.1. Collect the used Hyb-, Hyb+, and probe solutions in a separate waste container for proper disposal.

8.5. Replace the Hyb- with 4 mL of the Hyb+ solution.

8.6. Incubate the vial at 70 °C overnight.

8.7. Dilute the EGFP-fluorescein probe 1:100 in 500 µL of Hyb+ and incubate it O/N at 70 °C.

NOTE: Follow the manufacturer's instructions for probe synthesis.

9. Day 6: Probe hybridization

9.1. Replace the Hyb+ solution in the vial with the preheated probe and incubate at 70 °C overnight.

NOTE: Larvae longer than 6 mm need 2 days of incubation.

9.2. Preheat the wash buffer, 2x SSCT, and 0.2x SSCT solutions at 70 °C for overnight.

10. Day 7: Probe washing

10.1. Replace the probe with 4 mL of the preheated wash buffer and incubate at 70 °C for 30 min.

10.1.1. Collect the used probe in a separate waste container for proper disposal.

10.2. Replace the wash buffer with 4 mL of fresh wash buffer and incubate at 70 °C for 30 min.

10.3. Replace the wash buffer with 4 mL of the preheated 2x SSCT solution and incubate it at 70 °C for 15 min.

10.4. Add 50 mL of preheated 0.2x SSCT into a 50 mL tube.

10.4.1. Insert a cell strainer (100 µm) into the top of the tube. Ensure that the cell strainer is

pushed all the way down until it stops.

10.4.2. Transfer the larvae from the glass vial into the cell strainer. Ensure that the larvae are submerged in the buffer and incubate at 70 °C for 2 h.

10.5. Prepare a new tube containing 50 mL of preheated 0.2x SSCT.

10.5.1. Transfer the cell strainer from step 10.4.2 into the new tube of 0.2x SSCT and incubate at 70 °C for 2 h.

10.5.2. Repeat step 10.5 one more time.

11. Day 7: Blocking

11.1. Transfer the larvae to a new glass vial and cool to room temperature.

11.2. Replace the 0.2x SSCT with 4 mL of 67% 0.2x SSCT/33% MABT and rock at room temperature for 10 min.

11.3. Replace the solution in step 11.2 with 4 mL of 33% 0.2x SSCT/67% MABT and rock for 10 min.

11.4. Replace the solution in step 11.3 with 4 mL of MABT and rock for 10 min.

11.5. Replace the MABT with 4 mL of fresh MABT and rock for 10 min. Repeat one more time.

11.6. Replace the MABT with 4 mL of the blocking solution and incubate at 4 °C overnight. It is possible to incubate longer for convenience.

12. Day 8–9: Antibody incubation

12.1. Dilute the anti-fluorescein antibody 1:5,000 in 500 µL blocking solution.

12.2. Replace the blocking solution with the antibody solution and incubate at 4 °C for 2 days. It is possible to incubate longer for convenience.

12.2.1. Swirl the vial twice a day to agitate the larvae.

NOTE: Larvae longer than 6 mm need 1–2 more days of incubation.

13. Day 10–11: Antibody washing

13.1. Replace the antibody solution with 4 mL of PBST and rock at room temperature for 10 min.

13.2. Replace the PBST with 4 mL of fresh PBST and rock for 10 min. Repeat one more time.

13.3. Transfer the larvae into a 50 mL tube.

13.4. Add 40 mL of PBST2. Lay the tube on its side and rock at 4 °C overnight.

13.5. Replace the PBST2 with 40 mL of fresh PBST2 and rock at 4 °C overnight. It is possible to incubate longer for convenience.

NOTE: Larvae longer than 6 mm need 1–2 more days of washing.

14. Day 12: Staining

14.1. Transfer the larvae to a 6-well plate.

14.2. Replace the PBST2 with 3 mL of staining buffer and rock at room temperature for 5 min.

14.3. Replace the staining buffer with 3 mL of fresh staining buffer and rock for 5 min. Repeat one more time.

14.4. Replace the staining buffer with 3 mL of the staining solution. Cover with aluminum foil and monitor the staining over the next few hours.

14.4.1. For probes with a weak signal, incubate at 4 °C overnight and replace the staining solution with fresh staining solution once in between.

14.5. When the desired staining intensity is reached, replace the staining solution with 3 mL of the stopping solution and rock for 30 min.

14.6. Replace the stopping solution with 3 mL of fresh stopping solution and rock for 30 min. Repeat one more time.

14.7. Transfer the larvae to a new glass vial and replace the stopping solution with 4 mL of fresh fixing solution and incubate for 1 h at room temperature. It is possible to incubate longer at 4 °C for convenience.

14.8. Store the larvae in the fixing solution in the dark at 4 °C for up to a year.

15. Day 12: Imaging

15.1. Replace the fixing solution with 4 mL of PBST and rock at room temperature for 10 min.

15.2. Transfer the larvae to a 6-well plate.

15.3. Replace the PBST with 4 mL of fresh PBST and rock for 10 min. Repeat one more time.

15.4. Replace the PBST with 3 mL of 50% glycerol (in PBST) and rock for 10 min.

15.5. Replace the solution in step 15.4 with 3 mL of 100% glycerol with no rocking for 10 min.

15.6. Use a dissecting microscope to image the larvae directly in the 6-well plate or transfer the larvae into a depression slide to image under a compound microscope. Use an eyelash manipulator to orient the larvae.

REPRESENTATIVE RESULTS:

Using the *Tg(lhx1a-EGFP)* transgenic line, it was demonstrated that this *in situ* hybridization protocol is effective in labeling kidney progenitor cells and various nephron structures during mesonephros development. As expected, the central nervous system is also labeled in this transgenic line (not shown). The initial mesonephric nephron forms at approximately 5.2 mm, dorsal to the pronephros (**Figure 2A**), and the distal tubule of this nephron is labeled by EGFP[10,12]. Progenitor clusters are present at this stage and later on (**Figure 2A–B**, arrowheads), and single progenitor cells are also labeled (**Figure 2C**). This method provides an additional tool in studying kidney development and helps shed light on understanding the human kidney.

FIGURE AND TABLE LEGENDS:

Figure 1: Measuring larvae. Fixed larvae in a Petri plate are placed on top of a flat ruler. Under a dissecting microscope, the larvae are measured and separated by groups of similar sizes.

Figure 2: Mesonephros development. At around 5.2 mm in the *Tg(lhx1a-EGFP)* transgenic line, the first mesonephric nephron is formed dorsal to pronephros and swim bladder (SB) (**A**). Clusters of progenitor cells are present during mesonephros development (**A–B**, arrowheads) in addition to single progenitor cells (**C**, bracket). In larger juveniles, background staining can occur in the somites (**C**, arrows).

DISCUSSION:

The *in situ* hybridization method described here is aimed toward studying mesonephros development. However, it can be applied to study the development of other tissues and organs during metamorphosis, such as the gut, nervous system, scales, and pigmentation[14]. Probes can be generated for endogenous genes or fluorescent markers in transgenic lines.

It is critical for the larvae to remain intact in order to observe the organs and tissues in their native context. To retain tissue integrity, it is important to minimize the time of proteinase K treatment. It is important to determine the best treatment time for each new batch of enzyme. Alternatively, acetone can be used instead of proteinase K for improved tissue integrity[16]. Excessive bleaching also reduces tissue integrity. Minimal bleaching and allowing the eyes to retain some pigmentation help in visualizing the larvae during washes. It is common for the eyes to fall off during the hybridization step, which is an indicator of tissue fragility. The use of DMSO

with the fixing and proteinase K solution is crucial for tissue penetration[17].

A limitation of this method is the poor penetration of reagents in larger animals. To improve penetration, the head and tail can be removed with a razor blade before fixation[17]. The gut can be removed with fine tweezers and tungsten needles after fixation to allow direct access of the reagents to the mesonephros. Long probes (greater than 1 KB) can have poor penetration of the tissue, but they can be hydrolyzed into short fragments (around 0.3 KB) to improve penetration[18]. For probes with weak signals, control probes with the sense sequence can be used to differentiate between the background and the probe signal. Larger animals will have a higher background staining of the somites (**Figure 2C**, arrows). However, this can be minimized with longer washes of the probe and antibodies.

The protocol here can also be applied to dissected and isolated tissues and organs, such as the adult zebrafish kidney[12,19]. Although there are published descriptions of similar protocols[9,16], none of them describe the entire process from rearing larvae to *in situ* hybridization with this level of detail. Therefore, this method provides an additional tool in deciphering the development of the vertebrate kidney.

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DISCLOSURES:

The authors have no conflicts of interest.

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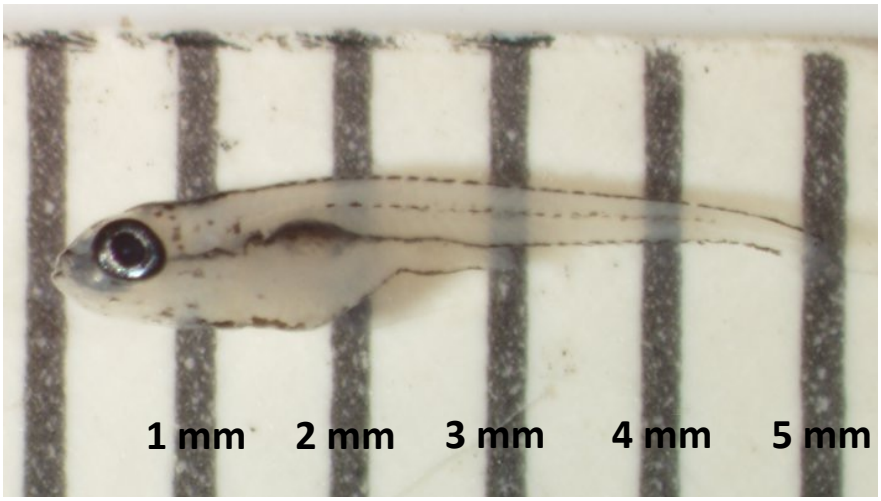
Figure 1



~5.2 mm larva

Figure 2

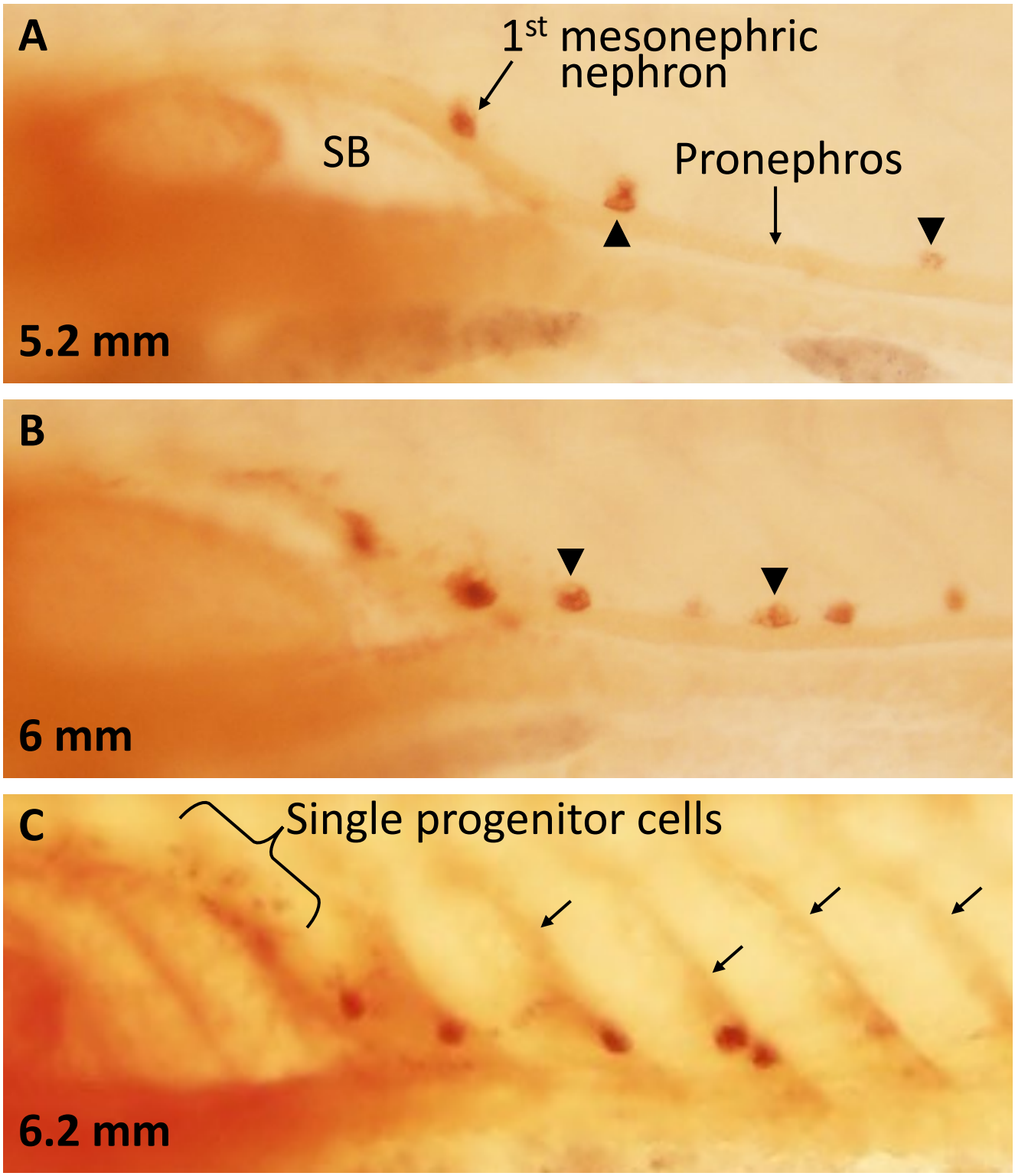


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Table of Materials

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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. Please define all abbreviations at first use.

Done

2. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Done

3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Done

4. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should

be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

A new NOTE was added in Step 1.1 of the R1 version.

5. As you have many solutions in your protocol, please provide solution composition as Tables in separate .xls or .xlsx files uploaded to your Editorial Manager account. These tables can then be referenced in the protocol text.

The solution compositions are listed in the “Comments” column of the Table of Materials. The following sentence was added before Step 1: “Details of solution content are listed in the Table of Materials.” Let us know if you want 2 separate tables for the Materials and the Solutions.

6. I) Lines 125 and 126: what is the concentration of tricaine?

Its concentration is listed in the Table of Materials, but we also added the composition to this line in the text.

ii) Line 132: which fixing solution, what is the concentration?

There’s only one fixing solution in the protocol. Its concentration is listed in the Table of Materials, but we also added the composition to this line in the text.

iii) Line 154: What is the capacity of the glass vial? How many larvae per vial? Per tube?

We added “5.5 mL” into this line. The protocol already stated “up to 10 larvae per vial.”

iv) Section 5: what is the composition of PBST?

This is listed in the Table of Materials.

v) Section 6: What is the concentration of proteinase K?

This is listed in the Table of Materials, but we also added it to this line.

vi) Line 193: how many larvae per well?

We added “up to 10 larvae per well.”

vii) What is the composition of the Hyb solution?

viii) What is the composition of SSCT, blocking solutions, staining solution and buffer?

This is listed in the Table of Materials. Let us know if you would like the details to be added to the protocol text.

7. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Done

8. Please sort the Materials Table alphabetically by the name of the material.

Fixed

Reviewer #1:

Manuscript Summary:

This is a very detailed protocol describing how to perform in situ hybridization on larval fish, with improved penetration of probes and antibody. I think it will be very useful for zebrafish labs.

Major Concerns:

I would suggest also breaking it up to days, or making the days from start to finish more clear, as it is hard to judge how long this will take overall, and how much time you need to allow and plan.

- We made the suggested changes.
- For Step 1 (Raising larvae), we added a "NOTE" above Step 1.1 to indicate that it could take up to 21 days or more to raise the larvae and juveniles.
- For the rest of the protocol, we added "Day 1:," "Day 3:," (etc.) to the appropriate steps to give a clearer indication of how long the entire process takes.
- Step 8 was split into 2 steps (Step 8 and Step 9) and indicated as "Day 5" and Day 6."

Minor Concerns:

1.2 point could be more concise. Is there a time in the afternoon that the authors suggest would be best?

We added 1 pm for the time.

1.2.3 how long do you incubate?

We added 1 day of incubation.

1.5 "400 µM" I assume should be 400 µm mesh screen?

Yes, this was fixed.

1.7.1 this point could be a Note as there seems to be no action.

Yes, this was fixed.

2.3 Clarify this point or perhaps reorder with the subpoints as it's hard to understand the sequence. The fish are not actually taken out initially correct? You keep them in the net towards the top?

Yes, this was fixed.

2.4 Concentration of tricaine? We added “2%, pH 7 stock”. Volume of E3 in the petri dish? We added “20 mL” in Step 2.2.

2.4.2 with the Optional point, is this after tricaine treatment?

Yes, we added “after euthanasia” to clarify this.

7.5 what should someone be looking for when reaching the right amount of bleaching?

We added “Look for the disappearance of pigmentation along the mesonephros (just above the swim bladder and gut).”

9.4 This seems like a large volume but you are only technically using the top of the tube, can it be done in a 6-well plate to reduce the volume of SSCT?

The large volume is needed to enhance washing of the probe. Although the larvae are laying towards the top of the tube, the mesh in the cell strainer allows for probe and SSCT diffusion throughout the entire 50 mL tube.

Reviewer #2:

Manuscript Summary:

This protocol describes in situ hybridization of mesonephric kidney tissue in juvenile zebrafish. This stage of zebrafish development is of interest for understanding how new kidney tubules form in the growing fish and how these new structures integrate into the existing embryonic form of the kidney. These insights are relevant for understanding and developing regenerative approaches in the mammalian kidney. The manuscript is well structured with sufficient introduction and discussion. Importantly, the described technique can be applied to other tissues and organs in juvenile zebrafish, thus making this protocol useful to a broad range of zebrafish developmental scientists than just the kidney experts. Also included in the discussion are the limitations of the protocol and a troubleshooting section that provides solutions for common technical problems. The materials and equipment list appears to be complete. This is also the case for the references. The steps in the procedure part of the manuscript are logical and easy to follow. Critical steps are highlighted and controls are included. Overall, this protocol is well written and provides a useful contribution to the field. I recommend it for publication in JoVE. I only have minor comments and suggestions to be addressed.

Major Concerns:

I have no major concerns.

Minor Concerns:

1. Line 108, 400 µm, not µM fixed

2. Figure 1, maybe include the numbers on the ruler fixed

3. Line 176, maybe name this subheading 'Proteinase K digest' fixed

4. Lines 179-181, could the authors provide a table or examples of adjusted proteinase K digestion time or concentration for longer larvae?

The following was added: “Start with a 10 min increase in incubation time for every 0.5 mm longer than 6 mm.”

5. Lines 189-192, step 7.2 is redundant with 7.1., could you just say wash with PBST 3 times?

The last sentence in Step 7.1 was deleted to make it not redundant.

6. Line 210, a wash step is missing here, i.e. replacing the fixing solution with PBST and wash x times

Step 8.1 and 8.2 were added to include this missing part.

7. Line 216, should it be mentioned how the probe is synthesized?

The following note was added: “NOTE: Follow the manufacturer’s instructions for probe synthesis.”

8. Line 229, step 9.1.1., does the collection of used Hyb solution also apply to step 8.3?

Yes. This step was moved up to where the Hyb solution was first mentioned.

9. Line 236, step 9.4.1, please include the size of the cell strainer

This was added.

10. Lines 237, 238, step 9.4.3 should be included into 9.4.2: transfer larvae…, ensure they are submerged…, then incubate for 2hrs

Both of these steps were combined into one to make it clearer.

11. Lines 235-241, I assume the setup of these steps will be shown in the video? Maybe a figure showing this setup would be helpful in addition.

These steps will be shown in the video.

12. Lines 262 and 273, 6mm+ larvae will need a longer incubation time/longer washes… how much longer, how long max? Be provide more detail.

The sentence was modified to: “NOTE: Larvae longer than 6 mm will need 1-2 more days of incubation.”

13. Line 346, please mention how the gut can be removed, what tools should be used for that.

The following words were inserted into this sentence: “…with fine tweezers and tungsten needles…”

14. Lines 346, 347, please define 'long' probes and 'short' fragments. What's the ideal length of a probe? What happens if a probe is too long?

The sentence was modified to include these details: “Long probes (greater than 1 KB) can have poor penetration of the tissue, but they can be hydrolyzed into short fragments (around 0.3 KB) to improve penetration.”

Reviewer #3:

Manuscript Summary:

Overall, Diep et al. did an excellent job explaining the protocol for imaging the mesonephros via in situ hybridization in juvenile zebrafish. This protocol does fill a void in the literature, as most focus on embryonic stages. I respect the limitations elaborated by the authors. The representative results are clear and a reasonable representation.

INTRODUCTION - The introduction accurately summarizes kidney development both in the embryo and in the juvenile zebrafish. In addition, the usefulness for studying kidney development into the juvenile stage has been outlined. Furthermore, the very real limitations into studying kidney development post-embryonic stages were explained. I feel the Introduction was well written and complete.

PROTOCOL - The protocol goes into sufficient detail for each step of the procedure. Further elaboration is given when necessary. Steps were clear and concise. Each step was thoroughly explained. Any reagents were identified including the supplier. Recipes were included for more complex reagents.

REPRESENTATIVE RESULTS - The representative results appear to be a reasonable representation of possible results obtained. The included figures are necessary to indicate to the reader the expected results, and the figures are clear and easy to understand.

DISCUSSION - I very much appreciated this discussion. It indicated the multiple approaches that could be taken with this protocol; to examine more than mesonephros development. In addition, important aspects of the procedure were elaborated upon and reasonable limitations were explained.

Major Concerns:

I have no major concerns with this manuscript.

Minor Concerns:

In several points throughout the procedure (Step 6.1, 11.2, 12.5) it was indicated that if performing the procedure on larvae that are larger than 6mm, adjustments will need to be made with the protocol. However, no further details were given. Providing the reader with an idea of the degree of adjustment necessary (i.e. if the larvae is X size, then you will need to incubate X minutes longer, etc) would be beneficial. This was done sometimes (for example, Step 8.6), but it was not common. (we have addressed all of these concerns) Although not necessary to comment on grammar, there were instances of minor errors in grammar within the Abstract and Representative Results. (we have fixed all of the grammar errors)