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Embedding, Serial Sectioning and Staining of Zebrafish Embryos Using JB-4™ Resin

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Abstract

Histological techniques are critical for observing tissue and cellular morphology. Here, we outline our protocol for embedding, serial sectioning, staining, and visualizing zebrafish embryos embedded in JB-4™ plastic resin – a glycol methacrylate-based medium that results in excellent preservation of tissue morphology. In addition we describe our procedures for staining plastic sections with Toluidine Blue or Hematoxylin and Eosin (H&E), and show how to couple these stains with whole-mount RNA in situ hybridization. We also describe how to maintain and visualize immunofluorescence and GFP signals in JB-4™ resin. The protocol we outline – from embryo preparation, embedding, sectioning and staining to visualization - can be accomplished in three days. Overall, we reinforce that plastic embedding can provide higher resolution of cellular details and is a valuable tool for cellular and morphological studies in zebrafish.

Keywords

histology; zebrafish; JB4; methacrylate; embryos

Introduction

Histology is a classic technique that is used to study the morphology of cellular and sub-cellular structures by cutting specimens into thin sections. Even with real-time imaging technology, histological sectioning and staining remain necessary techniques to visualize cellular morphology in tissues. Histotechniques are important tools for understanding the progression of tissue development and addressing phenotypes related to mutant backgrounds or pathological states.

Zebrafish are an attractive model for studying tissue morphogenesis, owing in part to their optical clarity during embryonic stages. Sectioning through the entire embryo is feasible because embryos are small at embryonic and larval stages (around 1mm)[1]. Furthermore, bones have not matured at embryonic stages[2], so the tissue remains soft and provides less resistance when sectioning. Here, we present our optimized protocols for serial sectioning zebrafish embryos with JB-4™ plastic resin. Our protocol for embedding and sectioning zebrafish embryos in JB-4™ resin (Figure 1) has been adapted from the protocol originally provided for JB-4™ by Electron Microscopy Sciences and optimized for zebrafish embryos. Work from our laboratory has used these protocols to analyze kidney and heart development

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in zebrafish[3–8]. We also provide information on how to couple JB-4™ embedding with whole-mount RNA in situ hybridization, immunofluorescence and GFP fluorescence. Our protocol can be accomplished in three days as outlined in Figure 1.

Advantages and Disadvantages of Plastic Embedding

Depending on the type of analysis necessary for a given experiment, there are many histological techniques available, each having their own advantages and disadvantages. Among the most common techniques are frozen/cryoembedding, paraffin embedding, and plastic embedding for sectioning. For examples utilizing zebrafish embryos please see[9–11]. Cryoembedded samples do not need to be fixed prior to embedding so sections can be prepared quickly and epitopes are typically well preserved in the sample; however, the cellular morphology is generally poor due to the freezing process. Samples used for paraffin embedding require fixation before the embedding procedure and more tissue processing steps than cryoembedding; however, morphology is greatly improved. In both systems, immunofluorescence and RNA in situ hybridization can be performed after sectioning.

JB-4™ is a glycol methacrylate-based polymer used as embedding material that can be used to cut semi-thin or thicker sections. The main advantage to embedding in plastic resins, such as JB-4™, is the ability to easily produce ultra-thin (0.5–1uM) or semi-thin (2–3um) sections depending on the plastic embedding medium[12]. It has long been appreciated that thin sections provide an increased level of cytological detail over thicker sections. While embedding and sectioning in paraffin can provide reasonably good results, semi-thin sections are difficult to produce in paraffin, and the artifacts produced in wax can limit any improvement in cellular resolution that thinner sectioning would provide[12]. Thus we recommend our protocol for any analysis where preservation of cellular detail is critical. In addition, processing embryos for paraffin embedding takes many more steps and requires dehydration and clearing agents such as xylene which are often toxic and difficult to dispose of. While paraffin embedding can be done without special equipment, it is typically performed using processing machines which minimize exposure to toxic clearing agents, but may not be available to some users, or may process samples inefficiently. By contrast, plastic embedding requires no special processing equipment, can be performed with or without dehydration, and samples can be stored after embedding indefinitely if processing for morphology. Our protocol is also recommended when sectioning embryos that have previously been processed for RNA in situ hybridization or immunofluorescence, both of which can be altered by the multiple steps involved in paraffin processing.

While JB-4™ resin has some distinct advantageous in histology, RNA in situ hybridization and immunofluorescence must be performed in wholemount, prior to embedding and sectioning samples. Thus, our protocol is not recommended for analyses where these procedures would be more informative if performed following sectioning. Sample orientation in JB-4™ resin can also be challenging because plastic resins are initially water-like, unlike the viscous mediums in frozen and paraffin embedding. But, as we describe in this paper any desired orientation can be achieved utilizing multiple embedding steps.

Experimental Design

Using the protocol described in this paper, we have been successful in embedding and sectioning zebrafish embryos at multiple stages; from as early as 30% epiboly to adults. This protocol is recommended for any analysis where preservation of cellular detail is critical, or where sectioning of embryos is desired following wholemount RNA in situ hybridization or immunofluorescence. When performing sectioning following RNA in situ hybridization, best results are achieved with probes that provide strong signals since the amount of signal per section will be a fraction of the total stain. While embryos being processed for histology

can be stored indefinitely once embedded, embryos that are processed for RNA in situ hybridization or immunofluorescence should be sectioned and analyzed immediately after embedding to prevent a loss or reduction of signals. When analyzing mutant or morpholino injected embryos, it is important to process wildtype/control embryos in parallel to have samples for comparison to determine if any potential phenotypes are real, or an artifact of processing.

Materials

REAGENTS

- Zebrafish embryos – obtained by crossing adult zebrafish as described[11]. Embryos are maintained in a suitable growth media in petri dishes as described[11] until they are at the proper stage. We have used this protocol on embryos ranging from 6 hours post fertilization to adults.
- 4% Paraformaldehyde (pH 7.4) (Electron Microscopy Sciences, cat. #15713 or Sigma-Aldrich, cat. #158127)

  CAUTION - paraformaldehyde is considered toxic if swallowed, inhaled, or absorbed through the skin. (see REAGENT SETUP)
- Dent's Fixative (80% Methanol (MeOH) and 20% dimethyl sulfoxide (DMSO))
- PBS (Phosphate buffered saline)
- Ethanol, Absolute, 200 proof
- JB-4™ Solution A (Monomer) (Electron Microscopy Sciences, cat. #14270-01)

  CAUTION - Solution A contains acrylic monomer – n-Butoxyethanol which can act as an irritant if swallowed, inhaled, or absorbed through the skin. CRITICAL - note that we do not use JB-4 Plus™ in this protocol because it produces harder blocks that can shatter more easily
- Benzoyl Peroxide (JB4™ Catalyst) (Electron Microscopy Sciences, cat. #14270-06)

  CAUTION – benzoyl peroxide can act as an irritant if swallowed, inhaled, or absorbed through the skin.
- JB-4™ Solution B (Accelerator) (Electron Microscopy Sciences, #14270-04)

  CAUTION – Solution B contains N,N-Dimethylaniline which is highly toxic and irritating if swallowed, inhaled, absorbed through the skin or through eye contact. Be sure to use this solution in a ventilated fume hood.
- Gill's #2 Hemotoxylin Stain (Polysciences Inc., cat. #24243) or Hematoxylin (Fisher Scientific, cat. #H34525) (see REAGENT SETUP)
- Eosin Y (Electron Microscopy Sciences, cat. #14851) (see REAGENT SETUP)
- Acid water (120mM Hydrochloric acid (HCL) in dH_20)
- Scott's Tap Water Substitute (Fisher Scientific, cat. #ES8441)
- Sodium Bicarbonate ($NaHCO_3$) for Scott's Tap Water Substitute (Sigma-Aldrich cat. #S7277) (see REAGENT SETUP)
- Magnesium Sulfate ($MgSO_4 \cdot 7H_2O$) for Scott's Tap Water Substitute (Sigma-Aldrich Cat. #M2773) (see REAGENT SETUP)

- Toluidine Blue O (Sigma-Aldrich cat. #198151) (see REAGENT SETUP)
- Permount Mounting Media (Fisher Scientific, cat. #S70104)

EQUIPMENT

- RM2255 Rotary Microtome, Leica Microsystems
- Embedding Stubs, polyethylene, white, 4mm thin (Polysciences Inc, #23189)
- Embedding Molds, polyethylene, light microscopy, 12mm × 8mm (Polysciences Inc., #23184-1)
- Glass Knife Strips (6.4mm×25mm×400mm) (Electron Microscopy Sciences, #71012)
- GKM-2 Glass Knife Maker, RMC Products
- Forceps, Dumont #7, curved and fine points (Electron Microscopy Sciences, #72800)
- 10ml Syringe with 18 Gauge Needle for adding water drops to slides during sectioning.
- Glass Slides, Fisherbrand Super Frost Plus (Fisher Scientific, #12-550-15)
- Glass Staining Dishes with covers, holds 20 slides (Electron Microscopy Sciences, #70312-23)
- Glass Slide Rack, holds 20 slides (Electron Microscopy Sciences, #70312-24)
- Glass Cover Slips
- 50ml Polypropylene Conical Tubes
- 1.5ml plastic microcentrifuge tubes

REAGENT SETUP

Fixation

4% Paraformaldehyde (PFA) pH 7.4: Purchase as a powder (Sigma) or liquid (Electron Microscopy Sciences). If purchased as powder, dissolve 4g of PFA in 100mls of PBS, dissolve at 68, cool to room temperature (20–23°C) and adjust pH to 7.4. Paraformaldehyde solution can be purchased in different concentrations. We typically purchase 20% solutions and dilute to 4% in PBS. Store in aliquots at −20°C for up to 18 months. **CAUTION!** Work in a well-ventilated space and wear protective eye wear and gloves when handling PFA.

Embedding

Infiltrating Solution: Make according to the manufacturer's protocol. Add 0.625g of Benzoyl Peroxide (catalyst—provided with kit) to 50ml of JB-4™ Solution A (provided in kit). We make this solution in a 50ml conical tube and rock at room temperature for 20 min. or until dissolved. Store at 4°C in the dark for one month. **CAUTION!** Work in a well-ventilated space and wear protective eye wear and gloves when handling Benzoyl Peroxide.

Embedding Mixture: Make according to the manufacturer's protocol. Add 1ml of JB-4™ Solution B (accelerator-provided with kit) to 25ml of Infiltration Solution (prepared as described above). Make this solution in a 50ml conical tube, and mix by using a plastic pipette. This solution will begin to solidify quickly, so immediately add to the embedding molds. After adding it to all the molds, place the cap back on the conical tube and allow the remainder to polymerize. This will serve as a test to determine if the polymerization reaction

was successful. **CAUTION!** Work in a chemical hood and wear protective eye wear and gloves when handling all liquid components of JB-4™ resin.

Staining solutions—CRITICAL! Staining solutions cannot contain alcohol! Alcohol will cause the plastic sections to lose adherence to the glass slide.

Gill's #2 Hematoxylin – can purchase solution or make your own. Store at room temperature for up to 2 years.

	For 1L
1 Hematoxylin (0.4% weight/volume)	4g
2 Sodium Iodate (0.04% weight/volume)	0.4g
3 Aluminum Sulfate (3.5% weight/volume)	35g
4 Ethylene glycol (25%)	250ml
5 Acetic Acid (4%)	40ml
6 dH_20 to desired final amount	bring to 1L

Eosin-Y – Store at room temperature for up to 1 year.

For 1L	
1 Eosin-Y (0.5% weight/volume)	5g
2 dH_20 to desired final amount	bring to 1L

Scott's Tap Water Substitute – can purchase solution or make your own. Store at room temperature for up to 1 year.

	For 1L
1 Sodium Bicarbonate ($NaHCO_3$)	2g
2 Magnesium Sulfate Heptahydrate ($MgSO_4 \cdot 7H_2O$)	20g
3 dH_20 to desired final amount	bring to 1L

Scott's Tap Water Substitute is a “bluing” reagent at the correct pH to enhance the blue color of stained nuclei.

Toluidine blue solution – Store at room temperature for up to 18 months

	For 1L
1 Toluidine Blue O (1% weight/volume)	10g
2 dH_20 – to desired final amount	bring to 1L

PROCEDURE

Fixation and Storage

TIMING Variable (typically overnight at 4°C)

1 Fixation steps can vary depending on the sample and type of experiment. Here we describe typical fixation conditions for (A) morphological analysis, (B) RNA in situ hybridization or (C) immunofluorescence.

A. Fixation conditions for morphological analysis

i. Fix samples in a 1.5ml eppendorf tube with 4% PFA overnight at 4°C to achieve optimal tissue morphology. We typically add embryos up to the 200ul line on the

1.5ml eppendorf tube and add 1ml of fixative. Embryos can be stored in 4% PFA at 4°C for one week.

ii. After fixation, rinse embryos in 1× PBS (3 rinses, 5 min. per rinse).

CRITICAL STEP! For examining morphology, it is best to rinse in 1× PBS without a detergent. However, embryos are “sticky”, and will adhere to the sides of the tube. Gently flick the tube until the embryos settle to bottom.

iii. Embryos can be stored into PBS at 4°C for one week.

B. Fixation conditions for RNA in situ hybridization

i. Fix samples in a 1.5ml eppendorf tube with 4%PFA overnight at 4°C. We typically add embryos up to the 200ul line on the 1.5ml eppendorf tube and add 1ml of fixative. We then separate fixed embryos into tubes containing 30–40 embryos per tube for RNA in situ hybridization.

CRITICAL STEP! Fixed samples can be stored in PFA for one week; however, it is best to store in MeOH at −20°C for long term storage. If samples are in MeOH, *slowly* rehydrate them in PBS (see Step 2).

ii. After fixation, rinse embryos in 1× PBS (3 rinses, 5 min. per rinse).

iii. Continue with RNA in situ hybridization per standard protocols[13].

C. Fixation conditions for immunofluorescence

i. Fixation will depend upon the primary antibody. Typical fixations include 4% PFA or Dent's Fixative. We typically add embryos up to the 200ul line on the 1.5ml eppendorf tube and add 1ml of fixative. We then separate fixed embryos into tubes containing 10–15 per tube for immunofluorescence processing.

ii. If fixed in PFA, rinse embryos in PBS (3 rinses, 5 min. per rinse) and continue with immunofluorescence protocol.

iii. If fixed in Dent's Fixative, slowly rehydrate in PBS and continue with immunofluorescence protocol.

CRITICAL STEP! Embryos can be stored in Dents for at least one week at 4°C, but in general it is best to start the experiment soon after fixation.

Dehydration

2 For the best results, embryos should be dehydrated in EtOH before beginning the embedding procedure. Depending on the type of experiment, dehydration steps will vary. Here we describe typical dehydration conditions for (A)

morphological analysis, (B) RNA in situ hybridization or (C) immunofluorescence.

A. Dehydration conditions for morphological analysis **TIMING** 2 hours

i. After embryos are rinsed in PBS, *slowly* dehydrate embryos in EtOH using a step-wise series at room temperature as follows:

a 95%PBS/5%EtOH, 5 min.

b 75%PBS/25%EtOH, 5 min.

c 50%PBS/50%EtOH, 5 min.

d 25%PBS/75%EtOH, 5 min.

f 100%EtOH, 1 min.

ii. After the final step, rinse embryos in 100% EtOH for 30 min., under rocking conditions, at room temperature.

iii. Repeat step ii

B. Dehydration conditions for RNA in situ hybridization

i. After embryos have gone through the RNA in situ hybridization process, post-fix in 4% PFA for 2 hours at room temp or overnight at 4°C, and rinse in PBS (as described in Step 1). *Slowly* dehydrate embryos in EtOH using the step-wise series as described in (Step 2Ai).

CRITICAL STEP! After embryos have gone through the RNA in situ hybridization process, they can be stored in MeOH at −20°C indefinitely without significant loss of stain. To process samples stored in MeOH, replace the MeOH with EtOH (3 rinses, 5 min. each step).

C. Dehydration conditions for immunofluorescence

i. After embryos have gone through the immunofluorescence process, rinse embryos in PBS (3 rinses, 5 min. each step)

ii. *Quickly* dehydrate embryos in EtOH. Change the washes (1. 95%PBS/5%EtOH, 2. 75%PBS/25%EtOH, 3. 50%PBS/50%EtOH, 4. 25%PBS/75%EtOH, 5. 100% EtOH) immediately without waiting 5 min.

iii. After the last wash of 100% EtOH, *quickly* rinse the embryos twice in EtOH and proceed *immediately* to the infiltration step.

CRITICAL STEP! Do not prolong washes or rock embryos in EtOH as described for Steps 2(A) and 2(B) as this may decrease fluorescence signal. Once dehydration steps are finished, proceed directly to the infiltration step. According to the manufacturer's protocol, the embedding steps can proceed without any dehydration steps; however, in our experience with zebrafish embryos, the

morphology is improved by first doing this quick dehydration in EtOH.

Infiltration

TIMING 1–2 hours, then overnight

3 Remove all EtOH from each tube and replace with 1ml of Infiltration solution. The embryos will float to the surface. Allow embryos to sink to the bottom of the tube.

CAUTION! Perform all infiltration steps in a chemical hood and wear protective eye wear and gloves. Left-over liquid infiltration solution must be deposed of in an appropriate manner according to your institution's guidelines.

4 After embryos fall to the bottom of the tube, replace the original solution with 1ml of fresh Infiltration solution for 1–2 hours at room temperature.

5 Replace infiltration solution again, and incubate overnight at room temperature.

CRITICAL STEP! If examining immunofluorescence or GFP, embryos should be stored at 4°C overnight in the dark.

Embedding

TIMING ~30–45 min

6 Before beginning the embedding process, replace old infiltrating solution on the samples with new infiltrating solution.

7 Using a plastic pipette, remove 1–5 embryos and place each into an embedding mold. The embryos should always be covered with the infiltrating solution to prevent drying. The inset in Figure 2A shows the embedding mold (clear) in a metal holder with a white embedding stub (often called chucks).

CRITICAL STEP! The JB-4™ polymerization reaction is sensitive to the type of molds used in this step. It is important that air bubbles not be introduced as these will inhibit polymerization of the resin. In our hands, the polyethylene embedding molds described in the equipment section provided the most consistent polymerization results.

8 Prepare the embedding solution as described in REAGENT SETUP.

9 Remove the residual infiltration solution from the embryos in the mold, and add the embedding solution so that the mold is filled.

CRITICAL STEP! If the mold is not filled with enough embedding solution, air bubbles will result when the embedding stubs are placed over the mold. Air bubbles will prevent the JB-4™ resin from solidifying.

10 Position embryos with a toothpick. For transverse or longitudinal sections, the embryos are positioned laterally. Embryos will naturally fall on their sides, but should be positioned parallel to one side of the mold to facilitate orienting the sample for sectioning. See **Box 1** for further details.

11 Place the embedding stubs over the mold and cover with plastic wrap. Let the embedding solution harden for a minimum of 2 hours (Figure 2A) at room temperature.

?TROUBLESHOOTING

CRITICAL STEP! If examining signals from immunofluorescence or GFP, resin should harden in the dark at 4°C because the polymerization reaction is exothermic, and may interfere with the fluorescence.

12 Once the resin has solidified, the embryos can be stored indefinitely if performing morphological analysis.

CRITICAL STEP! If examining staining from an RNA in situ hybridization, immunofluorescence signals, or GFP expression, the signal will last 1–2 weeks in the resin, but it is optimal to complete the analysis within the shortest time frame possible to minimize decreases in signal.

Anticipated Results

Toluidine blue stain (Figure 3A–C, G)—Toluidine blue is a metachromatic dye that stains cellular components such as the nuclei and cytoplasm various shades of blue and tissues such as the cartilage a pinkish color (probably due to its high proteoglycan content[14]). Toluidine blue staining procedures are quick, and only require a 1% Toluidine blue solution.

Haematoxylin and Eosin Stain (Figure 3D–F, H)—The purpose of a Haematoxylin and Eosin (H&E) stain is to broadly distinguish nuclear material from cytoplasmic material[15].

The Haematoxylin is a basic dye with a high affinity towards basophilic structures (such as nucleic acids, which are concentrated in the nucleus) and yields a dark purple stain. The Eosin is an acidic dye with a high affinity towards eosinophilic structures (such as the protein rich cytoplasm) and yields a pink stain[15]. The dark purple nuclei have better contrast with the pink cytolplasmic stain than the toluidine blue stain; however the cartilage is not as easily identifiable.

Sample Distortion (Figure 3I–J)—Any methods involving fixation, processing and sectioning can result in shrinkage or swelling of the sample. Improper sectioning can further stretch and distort tissues. However we find that embryos processed correctly with this protocol show little distortion as measured by notochord cell diameter. These cells are highly vacuolated when mature, and can serve as a strong indicator of shrinkage in a tissue. The diameter of the mature notochord is ~50μm[16]. The notochord diameter was measured in two different images using ImageJ software and found to be 56 and 59μm which may indicate a minimal amount of swelling.

RNA Expression Pattern (Figure 4A–C)—RNA in situ hybridization is typically performed by standard methods in whole-mount zebrafish embryos[13]. However, it is often difficult is determine precise tissue localization of the RNA expression. Since staining from either 3,3'-Diaminobenzidine (DAB) or BCIP/NBT substrate applications is maintained in JB-4™ resin, histological analysis can drastically improve the cellular resolution of RNA expression. It is often desirable to take a picture of the sections without an H&E stain, as this stain will obscure weaker DAB or BCIP/NBT stains. However, when the in situ stain is strong (as in Figure 4C), the RNA expression domains are clearly distinguished from the H&E stain.

Protein Expression (Figure 4D–F)—In analyzing protein expression, determining cellular localization is often desired in addition to tissue localization. Although confocal microscopy can provide optical sectioning through samples, zebrafish embryos are often too thick for good resolution deep into the tissue in whole-mount, and morphology cannot be

appropriately analyzed. Therefore, sectioning is often critical to examine cellular localization of proteins. An example of a section through an immunostained embryo in whole mount is pictured in Figure 4D. JB-4™ resin can also be used to analyze GFP signals in sections – from both mosaically expressing (Figure 4E) and transgenic (Figure 4F) fish.

JB-4™ resin-based histology beautifully maintains morphology, enabling a close examination of cellular phenotypes, RNA expression, and protein localization. Although there are some challenges to using JB-4™ resin, the high level of tissue preservation and cellular resolution make it ideal for analysis of zebrafish morphology.

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Box 1. Embedding for Orientation

TIMING ~1–3 hours

One of the most critical steps in histology is obtaining proper orientation of the sample. The JB-4™ resin embedding solution is initially water-like, thus positioning embryos is difficult compared to using more viscous embedding solutions such as those used in cryoembedding. Although it is possible to embed a large number of samples and use the embryos that by chance are in the best orientation, we prefer to use multiple embedding steps to get a consistent and desired orientation and waste fewer samples.

A. Longitudinal Orientation: This is a one-step procedure. Orient the embryo laterally in the mold and use a toothpick to gently push the embryo onto its side. Add the embedding solution and embedding stub and let harden. Examples of embryos sectioned in as longitudinal orientation are shown in Figure 3G,H.

B. Transverse Orientation: This is a two-step procedure. First, embed the embryo as described for the longitudinal orientation. Once solidified, the resin block is detached from the embedding stub using a coping saw (Figure 2B). Alternatively, pull off the resin block from the embedding stub using an adjustable wrench. Identify the embryo by looking at the resin block through a dissecting microscope. Cut the resin block using a razor blade, making a smaller block that contains the embryo. Re-embed the smaller block by placing it in the mold in the desired orientation and follow previous instructions for embedding. Examples of embryos sectioned in a transverse orientation are shown in Figure 3A–F.

Sectioning TIMING ~ 1–1.5hours/sample

13 Shape the face of the resin block into a raised trapezoid for sectioning (Figure 2C). Using a razor blade, cut the resin (in small slivers at a time) so that the face of the block is in the shape of a trapezoid (Figure 2C).

CRITICAL STEP! Use a glass knife when sectioning as metal blades cause severe wrinkling of the sections. Glass knives are inexpensive; however, they will only last for 1–3 samples as the glass will chip and cause scratch marks on the resin.

14 Lock the embedding stub to the microtome and begin to trim the excess resin (Figure 2D). We typically trim at 6–10μm. In our experience, attempting to trim with thicker sections increases the chance that the resin will shatter. Stop trimming when the tissue sample is reached. The tissue can be seen in individual sections without a microscope by holding the individual plastic section up to a light.

?TROUBLESHOOTING

15 Collect the first few sections with sample and confirm that the sample is in the desired orientation by checking the sections under a dissecting microscope. Section collection can be challenging. The method that works best for us is to first place small droplets of water using a needle and syringe on a microscope slide (Figure 2E). Place the sections one at a time on each droplet of water on the slide, so that every section is collected in a consecutive order. To collect the sections, allow the section to rest on the glass knife. Using forceps, pick up the section at a corner (avoid scraping the edge of the glass knife with your forceps) and gently release the section on the water droplet (Figure 2F). Do not let the forceps touch the water, as the

section will quickly wrap around the forceps. The section should unwrinkle and flatten on the water.

?TROUBLESHOOTING

CRITICAL STEP! For beginners, we would suggest placing 2 rows of 3 droplets of water (6 total). Once the person is comfortable with collection samples, 3 rows of 9 droplets of water (27 total) can be used.

16 Adjust the angles and tilt of the knife and resin block to refine the position of the sample. Check the sample sections under the microscope periodically throughout the sectioning process to ensure that the sample is being cut evenly.

?TROUBLESHOOTING

17 Start to section and collect the samples. We typically section at 4μm.

18 After you have collected a slide of samples, dry sections on the slide at room temperature or on a hot plate at a low setting to quickly evaporate the water.

CRITICAL STEP! If examining embryos processed for RNA in situ hybridization, immunofluorescence, or GFP, do not dry on a heat block as this can denature the signal. Let the water evaporate at room temperature in the dark.

Slide Preparation TIMING ~ 2 hours-Overnight

19 Adding a cover slip to the sample is an important step that will vary depending on the procedure used. Listed below are the steps used for (A) Morphological Analysis, (B) RNA in situ hybridization, (C) Immunofluorescence.

A. Slide preparation for morphological analysis:

i. Perform a histological stain such as Toluidine blue or Haematoxylin and Eosin stain (see Box 2).

ii. Add Permount to secure the cover slip, as this will help smooth any remaining wrinkles in the sections. Use a plastic pipette to distribute the Permount in a small but continuous streak horizontally across the slide glass. Then, starting at one side, hold the cover slip to the slide and slowly lay down the cover slip allowing the Permount to wick evenly across the samples.

CRITICAL STEP! Once Permount is added, the cover slip is permanently adhered to the slide and cannot be removed.

iii. Dry slides cover slip face up (not standing up) for a minimum of 2 hours to overnight before examining under the microscope. Allowing the Permount to harden will ensure that the mounting media is not transferred to microscope components when viewing the slides.

B. Slide preparation for RNA in situ hybridization:

i. It is best to first examine and document the RNA in situ stain in sections before beginning the staining

procedure (Figure 4B,C) This is because the Toluidine blue or Haematoxylin and Eosin (H&E) stain can occasionally mask a weak RNA in situ hybridization signal.Before staining, use standard laboratory tape to secure a cover slip onto the slide to examine and photograph the RNA in situ hybridization stain. Do not use Permount as this will prevent removal of the coverslip and subsequent histological staining. Keep track of which section you photographed.

ii. Remove the coverslip and perform desired stain (see **Box 2**).

iii. Follow instructions in Steps 19Aii-iii for adding Permount to the slides

C. Slide preparation for immunofluorescence:

i. Typically, no counterstaining procedure is warranted when examining sections with immunofluorescence or GFP. However, it is essential to secure the cover slip with a 50%glycerol/50%water solution. Add 50%glycerol/50%water solution to the slide.

ii. Place cover slip on slide and immediately turn the slide to an upright position on its edge.

iii. Drain excess solution by blotting the edge of the slide.

iv. Dry slides in the dark at 4°C overnight and image the next day.

CRITICAL STEP! Because samples have been dehydrated, it very important to store the slides in the glycerol solution overnight at 4°C. This rehydration step will allow for better visualization of the fluorescence signal.

20 Examine the resulting sections on an appropriate microscope.

?TROUBLESHOOTING

Box 2 - Histological Staining Methods

Staining procedures are performed many different ways. In our lab, we use glass staining dishes that will hold the dye and a glass slide rack that can be dipped into the dishes and transferred to new dishes with the appropriate solution. These are described in the Equipment section of this paper.

CAUTION! Wear protective gloves and lab coat.

A. Toluidine blue **TIMING** ~ 10 min./slide (can do multiple slides at once)

- **i.** Add a drop of 1%Toluidine Blue O staining solution to each section and incubate for 30–60 seconds at room temperature. To dispense the Toluidine Blue solution, add to a syringe and dispense through a syringe filter to ensure that any insoluble particles are not deposited on the slide.
- **ii.** Rinse with running tap water for 5 min.
- **iii.** Dry slides, either at room temperature or heated on a hot plate.
- **iv.** Coverslip with Permount as described in **19Aii-iii.

B. Haematoxylin and Eosin Stain **TIMING** ~ 30 min./20slides

- **i.** Dip slides in Gill's #2 Haematoxylin for 5 min.
- **ii.** Rinse the slides under running tap water for 2 min.
- **iii.** Destain using acid water for 20 seconds.
- **iv.** Rinse the slides under running tap water for 1 min.
- **v.** Dip slides in Scott's Tap Water Substitute for 45 seconds.
- **vi.** Rinse the slides under running tap water for 3 min.
- **vii.** Dip slides in the Eosin dye for 3 min.
- **viii.** Rinse the slides under running tap water for 5 min.
- **ix.** Dry slides at room temperature or heated on low settings on a hot plate.
- **x.** Coverslip with Permount as described in 19Aii-iii.

TIMING

A summary of the approximate time needed to complete each stage of the procedure is provided in a flow diagram presented in Figure 1.

TROUBLESHOOTING

Troubleshooting advice is provided in Table 1.

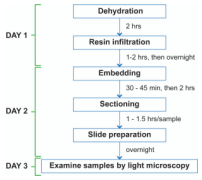

Figure 1.
Timeline of histological protocol using JB-4™ resin.

Figure 2.
Visual aid for embedding and sectioning zebrafish embryos in JB-4™ resin. (A) This is the set-up during the polymerization reaction of JB-4™ resin. A single polyethylene mold with a white plastic embedding stub in a metal casting tray covered with plastic wrap. The inset shows the mold and embedding stub before the specimen and JB-4™ resin are added. (B) During the second embedding step, the solid resin block is removed from the chuck with a coping saw. The chuck is first secured by a table vice before the resin block is removed. (C) Before sectioning, the resin block is trimmed with a razor blade so that the cutting surface will be in a trapezoid shape (trapezoid surface is traced with a gray line in the inset). (D) The set-up for sectioning on the Leica microtome. The embedding stub is clamped on the microtome. Below the embedding stub is the glass knife that is used for sectioning. (E) Before the sections are collected, rows of water droplets are placed on a glass slide with a syringe and needle. (F) Collect each section individually. Each section will stay on the glass knife after it is cut. The section is removed from the glass knife with forceps by gently picking it up at a corner and placing it on the water droplet.

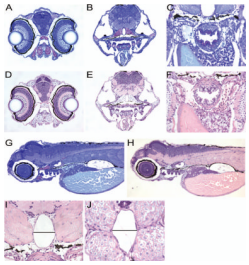

Figure 3.
Toluidine blue and Hematoxylin and Eosin staining on JB-4™ plastic sections. (A–C) Toulidine blue staining on transverse sections through the head (A), ear (B) and gut (C) regions of a 5 day wild-type embryo; Toulidine blue stains the nucleus a dark blue, the cytoplasm a light blue and the cartilage a dark pink color. (D–F) H&E staining on consecutive transverse sections through the head (A), ear (B) and gut (C) regions of the same 5 day wild-type embryo shown in A–C; H&E stains the nucleus a dark purple and the cytoplasm pink. (G–H) Consecutive longitudinal sections of a 4 day wild-type embryo stained with Toluidine blue (G) and H&E (H). (I–J) Sections through the notochord to show the typical amount of shrinkage/swelling seen with this procedure. The diameter indicated with the black line in each image was measured using ImageJ software. (I) Anterior notochord of a 5 day wild-type embryo. Notochord diameter is 56 microns. (J) Posterior notochord of a 2 day wild-type embryo. Notochord diameter is 59 microns. All sections are 4μm. A–B, D–E and G–H were taken were taken at 10× magnification, C,F were taken at 20× magnification. I was taken at 32× magnification. J was taken at 40× magnification.

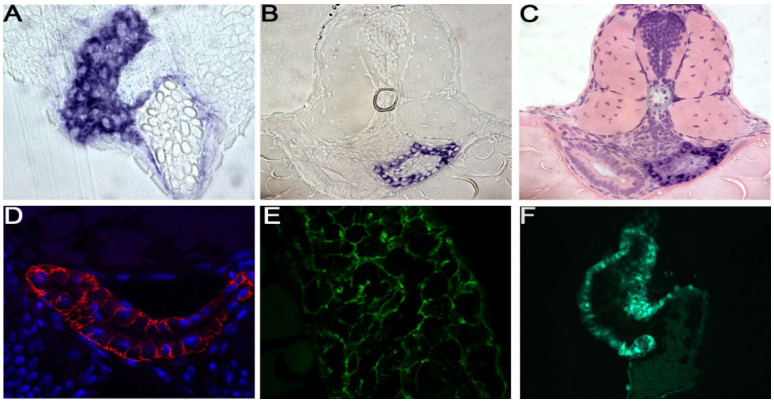

Figure 4.

RNA in situ hybridization, Immunofluorescence and GFP signals in JB-4 plastic resin. All images in this figure were produced using this procedure in the Burdine Laboratory. (A–C) RNA in situ hybridization developed with BCIP/NBT in whole-mount and subsequently embedded and sectioned in JB-4™ plastic resin. (A) *cmlc2* expression marks the myocardium of the atrial and ventricular chambers of the zebrafish heart[17]. (B) *carboxypeptidase A* expression marks the exocrine pancreas[18]. (C) Same section as shown in B, stained with an H&E stain. (D–F) Immunofluorescence and GFP visualization in JB-4™ sections. (D) Immunostaining for the sodium potassium pump (α6F antibody, red) and counterstained with Hoechst dye (blue), highlighting the cells of the zebrafish kidney tubule. At these stages the Na^+-K^+-ATPase pump is expressed in the pronephric duct and is clearly localized to the basolateral surface[6]. Embryos were fixed in Dent's. (E) RNA injection of GAP43-GFP to outline cell membranes[19]. Mosaic expression of GAP43-GFP is preserved in JB-4™ plastic sections at the 2 somite stage. The GFP signal is clearly seen surrounding the cell membranes in this section. (F) *cmlc2*-GFP transgenic zebrafish strain[20]. In this section, *cmlc2*-GFP is strongly expressing within the cells lining the ventricular chamber of a 2 day zebrafish heart.

Table 1

TROUBLE-SHOOTING

Step	Problem	Possible Reason	Solution
11	Resin is too soft and fails to polymerize completely	Air bubbles formed in the mold	Re-embed sample and be sure to use enough embedding media such that air bubbles are not introduced into the mold.
		Solutions were not mixed thoroughly	Mix solutions thoroughly and verify polymerization by allowing the unused portion to solidify in the 50ml conical tube.
14, 15 and 16	Resin is too hard leading to shattering of the block during sectioning.	Resin block was refrigerated	Ensure resin block is at room temp before sectioning
		Solutions are expired	Verify that solutions are not expired
		JB-4Plus was used instead of JB-4	Verify that JB-4 is used
15	Sections curl in on themselves when placed on to water drops on the slide	Resin is polymerized but still too soft	Refrigerate block for a minimum of 2 hours
		Sections are curling as they are being cut	Use the forceps to gently uncurl the section before taking it off the knife and placing it onto a water drop
		Sections are too thick >8um	We recommend 4um sections
16	Sections are not consistent in their size	Block is not secure in the microtome	Make sure the block and knife on the microtome are properly secured.
		Knife is dull	Try replacing the knife
		Resin is too hard or soft	If the microtome is `skipping' (ie. cutting a thicker or thinner section randomly), you can try to section through the block very slowly.
16 and 20	Scratch marks are observed in the resin	Knife is scratched	Either move the resin block to a different area of the knife or replace the knife
		Some scratch marks are expected and these may disappear once the sections are in mounting media	Add mounting media and coverslip as described in 19A
20	Morphology is poor	Incomplete fixation	Use longer fixation times
		MeOH containing fixatives can affect morphology	Try a non-MeOH based fixative if possible
20	RNA in situ stain disappears in the sections	Samples in resin too long	Limit the time the sample is in resin by doing the primary and secondary embedding steps in one day and section and analyze soon after.
		In situ hybridization stain is weak	This procedure works best with strong in situ stains. Try overstaining during the in situ procedure if using a weaker probe.
		Subsequent histological staining obscures the in situ hybridization signal.	Try documenting the in situ pattern in the sections prior to performing a subsequent histological staining procedure.
20	Fluorescence is difficult to image	Fluorescence has faded	Limit the time the sample is in resin by doing the primary and secondary embedding steps in one day, and section and stain soon after.
		Sections were not incubated in the 50%glycerol/50% water solution	Store slide (with coverslip) at 4°C overnight in the solution to rehydrate samples.