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# Studying Gene Expression in Whole Embryos by *in situ* Hybridization: A Peer-to-Peer Laboratory Guide

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**ABSTRACT** The extracellular matrix (ECM) plays an important role in cell to cell signaling pathways. Our goal is to provide a full laboratory guide for students to study gene expression in zebrafish embryos by *in situ* hybridization. Prior to our study, the laboratory had observed disorganized and shortened cilia in cells that are important for cell signaling in the pronephric duct and neural tube floor plate of the zebrafish embryo. Ciliogenesis depends on a master transcriptional regulator, *foxj1a*, whose mRNA expression can be monitored through *in situ* hybridization and microscopic imaging. Knockdown morpholino-injected, control mismatched morpholino-injected, and uninjected embryos were fixed to determine if *foxj1a* transcription is qualitatively affected by ECM gene knockdown. Our results showed that the knockdown embryos portrayed an inconsistent *foxj1a* signal strength along the length of the pronephric duct, when compared to analysis of control mismatched and wild-type uninjected embryos. We created this manuscript for other students to observe how ECM gene knockdown can affect *foxj1a* mRNA expression, but also to give them a guide to the tools they would need to explore their own genes of interest, in zebrafish or in many other organisms and tissues.

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## INTRODUCTION

### Purpose of this Lab Exercise

To introduce undergraduate students, either in an independent research lab or in a more structured course lab (genetics/developmental biology/molecular biology), to *in situ* hybridization, a method to assay gene expression. Students will be given tools to select a gene of interest, create digoxigenin-tagged riboprobes, and perform the *in situ* hybridization protocol. While the exercise is written for the zebrafish embryo model, *in situ* hybridization can be adapted to many other models, for example, studying processes of arm regeneration in sea stars or the types of glial supporting cells present during development of the fruit fly central nervous system (Czarkwiani, Dylus, & Oliveri, 2013; Ahn, Jeon, & Kim, 2014). State-of-the-art examination of multiple genes at the same time is an extension of the basic whole-mount *in situ* hybridization technique, illustrated for several organisms in this recent article (Choi et al., 2016).

### Our Experience

We are sophomores in the Augusta University Professional Medical Scholars Program (7 year program) who took part in the 2016 CURS Summer Scholars Program working in a laboratory that studied zebrafish embryonic development. We tested the hypothesis that an ECM protein regulates cilia formation via modulating transcription, and thus mRNA expression, of *foxj1a*, a "master regulator" of motile cilia synthesis (Yu, Ng, Habacher, & Roy, 2008). Knockdown (ECM morpholino-injected), Control A (5-bp mismatched MO-injected), and Control B (uninjected) embryos were fixed, hybridized, and stained to observe the *foxj1a* signal strengths in the pronephros for each type of embryo, and at different time points (Bill, Petzold, Clark, Schimmenti, & Ekker, 2009). We expected to see reduction or loss of *foxj1a* expression in the knockdown embryo pronephros, but not in either control. At points in the following sections, we will illustrate steps from our study.

## LABORATORY METHODS AND RESULTS

### 1. Using the ZFIN Database and GE Dharmacon Website

The Zebrafish Model Organism Database (ZFIN; https://zfin.org) provides a resource of information about genes expressed at different stages of development.[1] Work with your research mentor or lab instructor on what process you want to study, and what genes might be involved in that process. For zebrafish embryogenesis, you might select a gene known to be involved in a process like left-right patterning, or among genes identified by RT-PCR of expressed mRNAs isolated at a certain age during

[1] Example: For *foxj1a*, we could see expression data from published papers (https://zfin.org/ZDB-GENE-060929-1178), and the available clones that could be ordered (http://dharmacon.gelifesciences.com/non-mammalian-cdna-and-orf/zebrafish-cdnas-and-orfs/?sourceId=EntrezGene/767737).

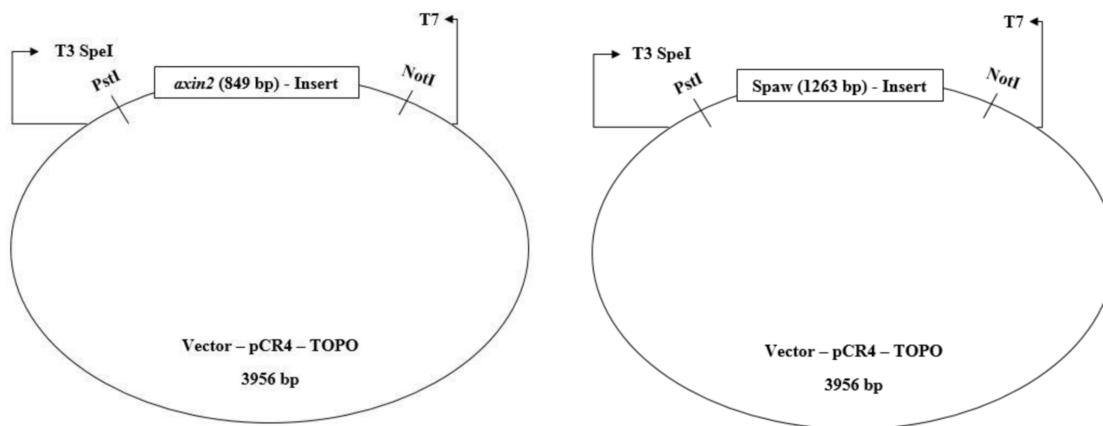


Figure 1. *axin2* (left) and *spaw* (right) plasmid maps used in restriction digests to determine the orientation of the cDNA inserts

development. Another important resource is the GE Dharmacon website, currently the only company that supplies cDNA clones created in the zebrafish genome project (http://dharmacon.gelifesciences.com/non-mammalian-cdna-and-orf/zebrafish-cdnas-and-orfs/?Parent=17179869650). Plasmid cDNAs generally provide a more reliable template for RNA probe generation than does attempting to amplify templates by RT-PCR from embryonic mRNA. The latter worked for us for *foxj1a*, but did not for two other genes, *axin2* and *spaw*, so we will next illustrate the methods you may need to utilize a plasmid cDNA received from GE Dharmacon.

## 2a. How to Make DNA template containing RNA polymerase binding site(s) using a plasmid

To generate single-stranded RNA probes, you will need a double-stranded, linear DNA template that contains a binding sequence for T3, T7, or SP6 RNA polymerase between 400-1200 base pairs 5′ to the end of a linearized DNA, with those >1000 bp performing best [3]. In many cases, genome project cDNAs were directionally cloned into vectors specifically designed to allow generation of sense and antisense RNA probes. Ideally, this plasmid map information is provided by GE Dharmacon site. Even if this information is provided, sometimes it is not clear in which direction the cDNA was put into the plasmid vector. If you have the vector but not the cDNA orientation within it, you can map the orientation by restriction enzyme digestion. A good rule of thumb is to select an enzyme that cuts once within the plasmid vector and once toward one end of the cDNA insertion; this will yield two DNA products per digestion, with very different predicted sizes for one orientation versus the other.

Figure 2. Digestion of axin2 and spaw plasmids, with comparison of resulting band sizes to markers.

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## Example

Based upon the two plasmid diagrams provided above, a strategy can be devised through restriction digestion to determine orientation of the *spaw* cDNA and axin2 cDNA in plasmid vector. We used the [NEBCutter v2.0 tool]() to analyze *axin2*, *spaw*, and pCR4-TOPO sequences. For the *axin2* plasmid, PstI was identified as cutting in the vector near the insert, and asymmetrically within *axin2* (position 1796 of 2645 total bases in the cDNA sequence). If *axin2* were oriented as shown in the diagram, the resulting fragments would be ~4805 bp (3956 + 849) and ~1796 bp; if it had the opposite orientation, the resulting fragments would be ~5692 (3956 + 1796) and ~849 bp. For the *spaw* plasmid, ClaI, KpaI, and HindIII cut asymmetrically within the insert but did not cut the vector. We determined that SpeI (cutting in the vector near the insert) and HindIII (position 919 of 1263 in *spaw* cDNA) could be used together in the same reaction tube to assess *spaw* cDNA orientation. If *spaw* were oriented as shown, the resulting fragments would be ~4300 bp (3956 + 344) and ~919 bp; if it had the opposite orientation, the resulting fragments would be ~4875 (3956 + 919) and ~344 bp. A gel run with the samples shows that both plasmids have the illustrated orientations (Figure 2).

## Protocol Example

Restriction digests to determine orientation of *axin2* and *spaw* in pCR4-TOPO vector.

| Reaction Component | Volume (µL) |
|---|---|
| $dH_2O$ | 5 |
| 10X NEB2 Buffer | 1 |
| 1 mg/ml BSA | 1 |
| (1µg) *spaw* plasmid | 1 |
| SpeI | 1 |
| HindIII | 1 |
| Total Volume | 10 µL |

Table 1. *axin2*- Cut with PstI (restriction enzyme). Incubate 37 °C for 90 min

| Reaction Component | Volume (µL) |
|---|---|
| $dH_2O$ | 6 |
| 10X NEB2 Buffer | 1 |
| 1 mg/ml BSA | 1 |
| (1µg) *axin2* plasmid | 1 |
| PstI | 1 |
| Total Volume | 10 µL |

Table 2. *spaw*- Cut with SpeI + HindIII. Incubate 37 °C for 90 min

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1. Add 2.5µL 5X DNA loading buffer, store at 4°C; also prepare DNA marker
2. Make 1.6% LE-agarose/TAE gel
3. Add 50 mL 1X TAE buffer to 250 mL volumetric flask
4. Add 0.8g LE-agarose powder
5. Microwave 30-60 seconds until boiling and all agarose powder is dissolved
6. Take out of microwave and let cool until you can hold base of flask comfortably
7. Add in 3 µL of EtBr solution [caution!]
8. Pour onto gel casting tray with 12-well comb, and allow to cool and harden for 30 minutes before loading samples.
9. Follow instructions for your DNA gel electrophoresis unit and power supply.

**2b. How to Make DNA template containing RNA polymerase binding site(s) using RT-PCR from total zebrafish RNA preparation**

This method can be used if you are unable to find a plasmid clone for your gene of interest, and know that it is expressed at a given time point. Here, isolate mRNA from your sample using Trizol reagent (Thermo Fisher Scientific Inc., 2016). Perform first-strand cDNA synthesis using oligo(dT) primers and transcriptase using a kit such as the SuperScript III First-Strand Synthesis for RT-PCR (Life Technologies Corporation, 2013). Instruction manuals for these steps are indicated in the References section. This gives potential synthesis of all mRNAs in the sample. Finally, a PCR reaction is set up as described below for *foxj1a*, which will amplify the desired sequence and add a T7 polymerase binding sequence that can be used for RNA probe generation in Section 3 (Thisse & Thisse, 2008). We isolated mRNA from 100 dechorionated 24 hour-old

zebrafish embryos and then generated first-strand cDNA using these protocols.

***Protocol Example***

After the above steps have given an ample amount of first-strand cDNA, a PCR reaction is set up for amplification of *foxj1a* template DNA. In each round of PCR, double-stranded DNAs are denatured with heat, then primers are allowed to re-anneal to single strands at lower temperature and extended. The reaction set-up is outlined in the second column below, and PCR machine program is outlined in the third column. Be sure to use tubes certified for use in your PCR machine.

| Reaction Component | Volume (μL) | Steps to program into PCR machine |
|---|---|---|
| $dH_20$ | 30 μL | 1 - 95 °C 3 min |
| 10X buffer | 5 μL | 2 - 50 °C |
| 10 mM dNTP | 1 μL | 3 - 72 °C |
| Forward primer (5 μM) | 5 μL | 4 - 95 °C |
| Reverse primer (5 μM) | 5 μL | 5 - 56 °C |
| DNA template (cDNA 24h) | 3 μL | 6 - 72 °C |
| Taq polymerase | 1 μL | 7 - Repeat from step 4 for 34 more cycles |
| Total | 50 μL | 8 - 72 °C for 5 min; 9 - hold in 4 °C |

Table 3. PCR Set-Up and Reaction Conditions

Primers used in experiment:

FoxJ1aF- 5′-CACTTTCATGCATCTACAAGTGG-3′FoxJ1aRT7- 5′-GAAGGTAATACGACTCACTCACTATAGGGTGAGGCCGAGGAAGTCGGAACG-3′

The product was run on a 1.2% GTG-agarose/TAE gel (Figure 3), and the major band cut out and purified using QIAGEN gel extraction kit using standard instructions except that 30 μl RNase-free $dH_2O$ was used to elute the DNA (QIAGEN, 2015). The amount of DNA template is estimated by comparison to known quantities of DNA in bands of the marker lane, or with a sensitive UV spectrophotometer

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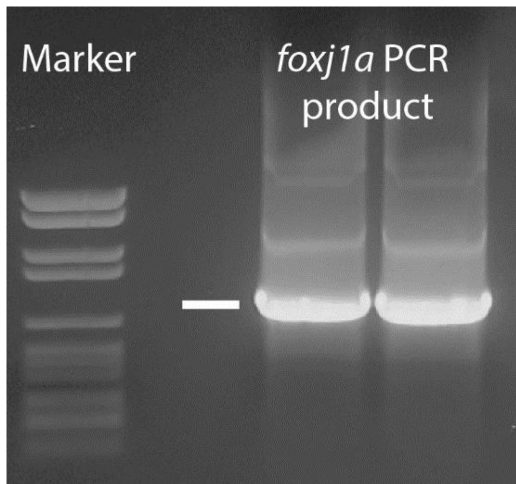


Figure 3. DNA template from PCR with T7 polymerase binding site at 3′ end; Band: *foxj1a*, 800 bp

### 3. ***In situ*** **hybridization probe synthesis**

Now that you have a double-stranded DNA template with an RNA polymerase binding site sequence 5′ to the sequence you want to amplify, you are ready to synthesize a single-stranded RNA probe (riboprobe). In this procedure, we only describe making antisense probes, that is, probes that are complementary to the mRNA that will be found in the embryo. In your experiments, you may also wish to make a sense probe that is a partial copy of the mRNA itself; in the *spaw* or *axin2* plasmids (Figure 1), this would involve cutting with a restriction enzyme that cuts close to the T7 polymerase binding site and then synthesizing riboprobe using T3 polymerase. Sense probes are used as negative controls for non-specific probe binding.

We used the Ambion MegaScript T7 Synthesis kit with addition of digoxigenin-11-UTP (DIGUTP-RO, Roche product sold by Sigma-Aldrich) (Life Technologies Corporation, 2012). The amount of unlabeled UTP should be adjusted downward to optimize labeling without compromising RNA yield; our laboratory advisor recommends a final 2:1 UTP: digoxigenin-11-UTP ratio.

**Protocol**

1. Thaw frozen reagents from Megascript kit (Ambion, AM1334, T7) and 10 mM digoxigenin-11-UTP on ice. Keep 10X reaction buffer at RT, because spermidine in buffer can coprecipitate template DNA if reaction is assembled on ice. (See Table 4)
2. Set up probe synthesis reactions at RT
3. Mix thoroughly
4. Incubate at 37 °C for 3 hr using PCR machine
5. Test 1 μL on agarose gel
6. Add 1 μL TURBO Dnase, mix well and incubate for 15 min at 37 °C

| Reaction Component | Volume (µL) |
| --- | --- |
| $dH_2O$ | Volume Needed for Final total 20 µL |
| 75 mM ATP | 2 µL |
| 75 mM CTP | 2 µL |
| 75 mM GTP | 2 µL |
| 75 mM UTP | 1.33 µL |
| 10X Reaction buffer | 2 µL |
| Linear template DNA | 0.1-1 µg (2-3 µL) |
| Enzyme mix | 2 µL |
| 10 mM digoxigenin-11-UTP | 5 µL |

Table 4. Reaction set-up for making riboprobe.

Recovery of DNA:

1. Stop reaction and precipitate the RNA by adding 30 µL Nuclease-free water and 30 µL LiCl precipitation solution
2. Mix thoroughly. Chill for >30 min at -20 °C
3. Centrifuge at 4 °C for 15 min at maximum speed to pellet the RNA
4. Carefully remove supernatant. Wash pellet with 500 µL 70% ethanol. Re-centrifuge to maximize removal of unincorporated nucleotides
5. Carefully remove 70% ethanol and air dry 5 min.
6. Dissolve RNA probe in 10 µL Nuclease-free water, and store in aliquots at -80 °C
7. Measure RNA (1 µL) by UV spectrophotometer.
8. Run on a gel to ensure there is a single prominent band, without smearing at faster positions that would indicate probe degradation (see Figure 4 for *foxj1a* probe example)

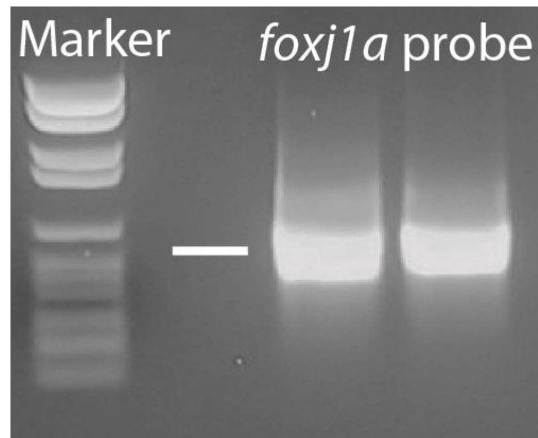


Figure 4. Synthesized DIG-UTP RNA antisense probe (T7), *foxj1a* RNA antisense probe, 4510.4 ug/mL

**4. Harvesting embryos, with prior MO injection if needed**

If you are using zebrafish or other vertebrate animals, IACUC approval is required for the laboratory from which you obtain embryo samples for *in situ* hybridization studies; if you use invertebrate samples such as fruit fly embryos or sea stars, IACUC approval is not necessary [1][2]. If you are going to handle live zebrafish embryos, or adult fish, at any point, you will need to go through appropriate animal use training as required by your institution. Although we went through this training in the course of our summer study, the laboratory's Research Associate, Hannah Neiswender, injected and later fixed zebrafish embryos while we observed. Embryos can be roughly staged by time after fertilization, but published images of embryos at specific developmental time points are helpful in accurately determining when you should fix embryos (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Fixation is with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), at 4 °C overnight in a microfuge tube on a rocker. While for many class labs, you may be looking only at mRNA expression patterns in wild-type embryos, in a research lab you may get to perform injections of MOs or CRISPR/Cas9 reagents. Rosen, Sweeney, & Mably, (2009) provide an overview of injecting zebrafish embryos in a video article.

**5. *In situ* hybridization**

Using quantitative real-time RT-PCR, reverse transcriptase polymerase chain reaction, with constant monitoring of fluorescent DNA-binding dyes during the PCR, the amount of gene expressed throughout an embryo can be determined. However, this procedure does not allow for the localization of which cells are expressing the gene. *In situ* hybridization provides a way to observe the level of gene expression arising in certain areas of the zebrafish embryo's body. Although qualitative, *in situ* hybridization allows for superior tracing of expression through the course of time in all the tissues of the growing embryo. The following *in situ* hybridization

protocol is based on that described by Thisse and Thisse (2008). It occurs over the course of three days, and should not be interrupted except at the indicated stopping points for each day.

Day 1:
Buffers needed:

1. DEPC-PBS

| Ingredient | Volume |
|---|---|
| PBS | 1000 mL |
| DEPC | 1 mL |

Table 5. Shake several times in a chemical hood at room temperature and leave it overnight in the chemical hood, then autoclave the next morning and keep at 4 °C

2. PBST (0.1%)

| Ingredient | Volume |
|---|---|
| Autoclaved DEPC-PBS | 1000 mL |
| Tween 20 | 1 mL |

Table 6. Add Tween-20 to DEPC-PBS while stirring with stir bar, keep at 4 °C.

3. 16% PFA

| Ingredient | Amount |
|---|---|
| PFA | 16 grams |
| PBS | 100 mL |

Table 7. Heat to 70°C for about 2 hours to dissolve the paraformaldehyde; Make 1 ml aliquots and keep at -70°C

4. 4% PFA

| Ingredient | Volume |
|---|---|
| 16% PFA | 1 mL |
| Autoclaved DEPC-PBS | 3 mL |

Table 8. This solution is made up fresh each day it is needed for fixing embryos; keep at 4 °C.

5. Hybridization buffer (HYB)

| Stock Solution | Volume | Final Concentration |
|---|---|---|
| Formamide (Sigma, F9037) | 25 mL | 50-60% |
| 20X SSC | 12.5 mL | 5X |
| Heparin (10 mg/mL) | 50 µL | 50 µg/mL |
| tRNA (10 mg/mL) | 3.5 mL | 500 µg/mL |
| 20% Tween 20 | 250 µL | 0.1% |
| 1M Citric Acid | 460 µL | To pH 6.0 |
| $dH_2O$ | 8.24 mL | |

Table 9. HYB buffer preparation; aliquots should be stored at -20°C.

The incubator should be preheated to 70 °C before the rehydration stage begins. During rehydration, the embryos, usually 10 to 20, are placed in a 700 μl microfuge tube. Setting a standard number of embryos in each tube allows for the determination of any lost embryos during the staining stage of *in situ* hybridization.

1. Rehydration: Put embryos (10 embryos) into 700 μL tube.
   a. 75% Methanol in PBS (750 μL Methanol, 250 μL PBS) 5 min at RT
   b. Discard supernatant after embryos settle when tube placed upright in a rack.
   c. 50% Methanol in PBS (500 μL Methanol, 500 μL PBS) 5 min at RT
   d. Discard supernatant
   e. 25% Methanol in PBS (250 μL Methanol, 750 μL PBS) 5 min at RT
   f. Discard supernatant
   g. Wash with PBST 5 min X 4 at RT on rocker, discarding supernatant each time

The PBST begins to permeabilize the tissue of the embryos. Make the Proteinase K solution during the last wash of PBST. Proteinase K opens up holes in the fixed embryo to allow for easier penetration of the riboprobe.

2. Digest with Proteinase K (10 μg/mL in PBST) at RT on a rocking platform (preferably nutator) for 15-30 min.

It should be noted that depending on the stage of the embryos (hpf) the concentration and time for digestion varies. This variation occurs due to the fact that younger stage embryos are more delicate and easier to penetrate, thus a lower concentration of Proteinase K is required, and a lower amount of digestion time is needed. Similarly, older stage embryos are tougher and harder to penetrate and thus a higher concentration of Proteinase K is needed and a higher amount of digestion time is required.

| **Embryo Age** | **Final Conc. Prot. K** | **Incubation time** | **Dilution of stock 20 mg/ml Proteinase K for the indicated final concentrations** |
|---|---|---|---|
| 17 hr | 10 μg/mL | 5 min | 0.75 μL 20 mg/mL Proteinase K into 1.5 mL PBST |
| 24 hr | 10 μg/mL | 15 min | 0.75 μL 20 mg/mL Proteinase K into 1.5 mL PBST |
| 48 hr | 25 μg/mL | 25-30 min | 1.88 μL 20 mg/mL Proteinase K into 1.5 mL PBST |
| 72 hr | 25 μg/mL | 25-30 min | 1.88 μL 20 mg/mL Proteinase K into 1.5 mL PBST |

Table 10. Proteinase K incubations based on age of embryos.

3. Refix embryos with 4% PFA-PBS for 20 minutes at RT (PFA is toxic, dispose in designated aldehydes waste container)
4. Wash with PBST 5X for 5 minutes each at RT on a rocker, discarding supernatant each time after embryos settle.
5. The hybridization buffer should be put at 70 °C to pre-warm.
6. Prehybridization: pre-hybridize embryos in 400 µL of hybridization mix for 4 hours at 70 °C.
7. The prehybridization buffer should be discarded, and 200 µL of hybridization buffer and probe should be added. Hybridization will occur overnight at 70℃.
8. The anti-Digoxigenin-AP antibody should be pre-absorbed through incubation with fixed embryos on a rocker, overnight at 4 °C. This pre-absorption is critical to reduce nonspecific binding during the staining of the embryos and the imaging of the results.

The ingredients for pre absorb the anti-DIG antibody:

- 12.5 µL of anti-DIG Fab fragment from Roche
    - + 5 mL blocking solution
    - + 500 fixed embryos (3 days old)

Day 2:

Incubator should still be at 70 °C, with your hybridization reaction tubes inside in a rack.

Buffers needed:

a. Anti-DIG-AP: Pre-absorb at 1:400 in Block solution and incubate overnight in 4 °C, then dilute in block solution to give 1:4000 final concentration.

| **Ingredient** | **Volume** | **Final Concentration** | **For 8 tubes** | **For 16 tubes** |
|---|---|---|---|---|
| BSA | 100 mg | [2 mg/ml] | 0.0125 g | 0.025 g |
| Sheep Serum | 1.0 mL | [2%] | 125 µL | 250 µL |
| DMSO | 1.0 mL | [2%] | 125 µL | 250 µL |
| 2% $NaN_3$ | 2.5 mL | [0.1%] | 63 µL | 125 µL (10%) |
| PBST | 45.5 mL | | 5.94 mL | 11.88 mL |
| Final Volume | 50 mL | | 6.253 mL | 12.5 mL |

Table 11. Preparation of Block solution.

b. Post hybridization mix (PHM)

| Stock solution | Volume | Final concentration | [4 tubes] | [8 tubes] |
|---|---|---|---|---|
| Formamide | 25.0 mL | [50-60%] | 2.125 mL | 7.5 mL |
| 20X SSC | 12.5 mL | [5X] | 1.565 mL | 3.76 mL |
| 20% Tween 20 | 250 µL | [0.1%] | 32 µL | 77 µL |
| 1M Citric Acid | 460 µL | [to pH 6.0] | 63 µL | 151 µL |
| $dH_2O$ | 11.79 mL | | 1.475 mL | 3.54 mL |
| Final Volume | 50 mL | | 6.26 L | |

Table 12. Preparation of post hybridization mix.

c. 2X SSC

| To make 2X SSC | [16 tubes] | [4 tubes] |
|---|---|---|
| 20X SSC | 5 mL | 1.25 mL |
| $dH_2O$ | 45 mL | 11.25 mL |

Table 13. Making 2X SSC

d. 0.2X SSC

| Solution | [16 tubes] | [4 tubes] | [8 tubes] |
|---|---|---|---|
| 2X SSC | 5 mL | 1.25 mL | 25 mL |
| $dH_2O$ | 45 mL | 11.25 mL | 22.5 mL |

Table 14. Making 0.2X SSC

Warm PHM and SSC solutions in 70 °C incubator.

Day 2 Protocol:

1. Post hybridization washes: Remove the probe mix from the embryos and wash with 100% post hybridization mix for 5 minutes at 70 °C. Remove the supernatant and discard in nonhazardous waste liquids container. Then, wash with:
   a. 75% PHM (750 µL) /25% 2X SSC (250 µL) 15 min at 70 °C
   b. 50% PHM (500 µL) /50% 2X SSC (500 µL) 15 min at 70 °C

c. 25% PHM (250 µL) /75% 2X SSC (750 µL) 15 min at 70 °C (After each wash, remove the supernatant and discard before adding next round)
d. 2X SSC for 15 minutes at 70 °C
e. 0.2X SSC for 30 minutes at 70 °C 2X (Removing supernatant and discarding is implied after each wash)
f. 75% 0.2X SSC/ 25% PBST 10 min at RT
g. 50% 0.2X SSC/ 50% PBST 10 min at RT
h. 25% 0.2X SSC/ 75% PBST 10 min at RT
i. PBST for 10 minutes at RT on a rocke
j. Incubate embryos in a blocking solution at RT for 3 hours on a rocker.

Preabsorbed anti-DIG (digoxigenin) antibody [1:400 during preabsorption] should be diluted to a final working solution that is 1:4000 in block buffer. Remove the supernatant in tube containing embryos and add in 500µL preabsorbed DIG ab.

Day 3:

Buffers needed:

1. AP Staining buffer

| Solution | Volume | Final [conc] | 4 tubes | 8 tubes |
|---|---|---|---|---|
| 1 M Tris-Cl pH 9.5 | 20 mL | [100 mM] | 1 mL | 2 mL |
| 1 M $MgCl_2$ | 10 mL | [50 mM] | 500 µL | 1 mL |
| 5M NaCl | 4 mL | [100 mM] | 200 µL | 400 µL |
| 20% Tween 20 | 2 mL | [0.2%] | 100 µL | 200 µL |
| $H_2O$ | 164 mL | | 8.2 mL | 16.4 mL |
| Final Solution | 200 mL | | 10 mL | 20 mL |

Table 15. Making AP (alkaline phosphatase) staining buffer.

2. 4% PFA

Protocol: For day 3, the antibody solution is removed and embryos washed with 400 µL volumes as indicated below.

1. Rinse with PBST at room temperature (very brief wash)
2. Wash with PBST at room temperature for 15 min 6 times – last two washes should be done on a rocker
3. Add AP staining buffer at room temperature for 5 min on a rocker – wash 3 times 5 min but move to a 24 well plate when 2nd wash with AP buffer

*6. Antibody staining/Fixation/Imaging*

Protocol:

1. After washes with AP buffer are complete, prepare NBT/BCIP reagent (Roche Applied Science, Cat # 1 681 451) as a 1:50 dilution in the AP Buffer. 400 μL of this AP staining solution are needed for each well.
2. The well plate is covered with foil because the color reagent is light sensitive, and will give increased background staining if exposed to light during this incubation. Incubation is on a rocker for around 5 minutes to 45 minutes at room temperature. This variable incubation time depends on the organism, and the concentration and specific behavior of the RNA probe used; it must be determined empirically for each new probe.
3. These embryos are then checked every 30 minutes to see when the optimal staining intensity has been reached for the pronephros of the embryos in the wild type and incubate at room temperature.

   Examples from our lab:
   a. *cmlc2* probe (100 ng or 400 ng riboprobe): nice staining appears at 30 minute incubation mark in the heart tube
   b. *dlx2a* probe (400 ng riboprobe): signal starts to appear on forebrain around 30 minutes and is complete around the 3.5 hour mark on the neural crest cell
   c. *tinagl1* probe (400 ng riboprobe): signal starts to appear after 1 hour and is complete at the 5.5 hour mark in the pronephros
4. Stop the reaction by removing the staining solution and washing the embryos with PBST four times for 5 minutes each
5. Rinse with PBS - if the staining is not fully complete place the embryos in PBS at 4°C overnight (not preferred), but if staining is done then fix embryos in 4% PFA for 2 hours at room temperature.
6. Wash 3 times with PBS to remove PFA and then store at 4°C in the dark
7. For observation after storage, using a dissecting microscope, mount embryos directly in 1% (or 2%) methylcellulose. Embryos can be repositioned in this drop of methylcellulose, and in most cases good photographs can be obtained.
   a. If needed, embryos can be flattened by removing the yolk with

forceps and then directly mounted on a slide under a coverslip with dots of vacuum grease at the corners to form a small chamber in which the embryo is not crushed.

8. Ideally, use microscope software and use forceps or a small tip brush/needle to move embryos around to see the different signals and take pictures. There are also now several microscope eyepiece adaptors that allow taking photos with your cell phone camera (e.g., https://www.amazon.com/iDu-LabCam-Microscope-Adapter-iPhone/dp/B00O98AHH0).

## OUTCOME AND DISCUSSION OF OUR *FOXJ1a* EXAMPLE

Our *foxj1a in situ* hybridization gave higher background than a control *cmlc2* riboprobe under the same conditions. In the future, it might be possible to test different amounts of *foxj1a* probe and increased number and stringency of washing steps to try to reduce this background. Notwithstanding this technical flaw, some conclusions could be made about the staining and effects of the ECM gene knockdown on *foxj1a* expression compared to wild-type embryo and mismatch MO controls.

As an end part of this experiment, the fixed embryos with the stained pronephric duct showed different results for each group in the experiment. For the wild-type control group, there were 12 wild-type embryos that were uninjected and used for imaging. All the embryos showed a consistent strength in the signal location and intensity of the staining along the pronephros and the CNS floor plate; however, there was a stronger-than-desired background staining present (Figure 5).

Figure 5: Wild-type Embryos

For the mismatch MO control group, there were a total of 10 embryos used for imaging purposes. This group showed a consistent staining pattern along the pronephric duct as seen for wild-type embryos (Figure 6). Since the RNA concentration was increased for this as well, the signaling was clear but the background staining was darker than usual as well.

For the experimental group where the embryos were injected with ECM

knockdown MO, there were 16 embryos used for imaging. These embryos showed a dark staining in the pronephric duct similar to that of the uninjected wild-type and mismatched morpholino embryos (Figure 7). However, there were inconsistent staining strengths along the pronephros region with the middle and anterior parts often showing a reduced or uneven intensity of *foxj1a* signal. The physical structure of the pronephros of the experimental MO knockdown embryos was affected to make it shorter, with an intense curvature for the pronephric duct and floor plate.

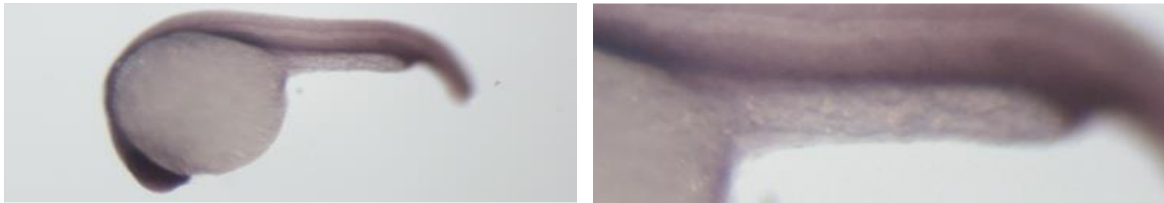

Figure 6: Control Mismatch MO Embryos

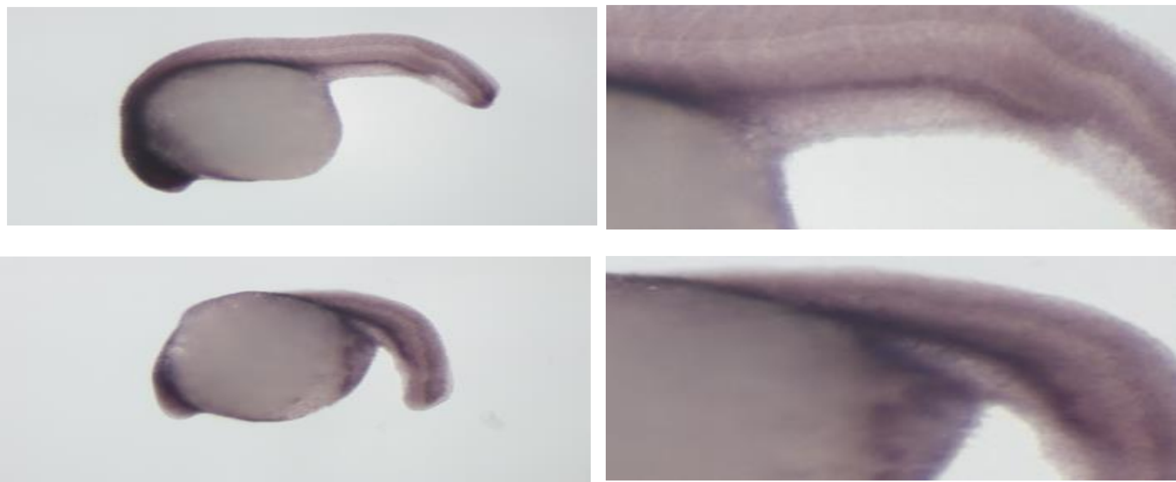

Figure 7. Experimental ECM knockdown MO embryos

Our results show that the morphant embryos portrayed an inconsistent and sometimes weaker signal in the pronephric region when compared to mismatched and wild-type uninjected embryos. We conclude that ECM gene knockdown does not result in an absolute loss of *foxj1a* mRNA expression, but changes may be occurring in pronephros differentiation or in regional alterations of motile ciliogenesis along the pronephros.

Possible errors may have occurred during these different experimentation processes. One error that could have affected the results obtained include the possibility that the concentration of RNA was increased too much to cause a darker

stained background which made it hard to see the signal in the pronephric duct and floor plate. Another error could have occurred during the process for *in situ* hybridization where NBT/BCIP reagent was added the solution of embryos; here, too much exposure to light may have affected the staining patterns causing there to be a change in the results acquired. The general conclusion can be made that ECM gene knockdown for *foxj1a* does not result in a complete loss of mRNA expression.

## SUMMARY

This lab exercise will provide students preparing to take a Molecular Biology Lab Course with first-hand experience with PCR and the preparation and use of gel electrophoresis. Students will also gain experience in the isolation of the DNA band upon separation of the DNA in gel electrophoresis. The procedures included in this lab exercise allow students to gain hands-on lab experience familiarizing them with fundamental research lab techniques that appear in molecular biology labs and set a strong foundation for those wishing to work in future research lab settings. Additionally, this lab exercise helps students in cell biology or microbiology by understanding techniques such as staining and fixation of specimens and viewing these organisms under different types of microscopes to see certain tissues and organelles if necessary. If students were to conduct this type of experimentation in zebrafish, they could also apply this research and knowledge to almost any clinical implications caused by ciliary malfunctions. Primary cilia are a very important cellular structure that play a major role in signaling pathways in the human body, which is analogous to the signaling seen in the zebrafish. If defects occur in cilia-associated signaling processes, it can cause complications such polycystic kidney disease. This kidney disease is caused by dysfunction in the primary, non-motile cilia found in vertebrate cells. Cysts, which are sacs that contain water-like fluid, inhibit the correct function of the kidney in the body, which then can lead to other complications and diseases. Viewing such a vast difference in the mutant zebrafish pronephros and the wild type zebrafish pronephros, can help students interested in the field of medicine think about the long term implications and problems that such mutations can cause to a human body. This lab exercise opens up a whole new horizon of questions that can be asked and that can lead to new innovations and experimental processes.

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