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# A fluorescence *in situ* hybridization (FISH) protocol for stickleback tissue

Noelle James[1], Xiaochen Liu[2] and Alison Bell[1,3]

[1]*Neuroscience Program, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA,* [2]*Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA and* [3]*School of Integrative Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA*

## ABSTRACT

**Background:** Threespine stickleback are an important model for behaviour and evolutionary studies. A growing number of quantitative trait loci (QTL) and gene expression studies are identifying genes related to ecologically important traits in sticklebacks. In order to visualize the expression of candidate genes, we developed a fluorescence *in situ* hybridization (FISH) protocol.

**Methods:** We present a protocol for FISH on fresh or flash-frozen dissected tissue, using either cryo- or paraffin embedding. The protocol covers probe design guidelines and synthesis, sample embedding, sectioning, and the hybridization process. The protocol is optimized for brain tissue. Key steps for modifying the protocol for other tissues are noted.

**Results:** The FISH protocol resulted in specific labelling under all combinations of dissection and embedding conditions. Paraffin embedding preserved morphology better than cryo-embedding. We provide representative results showing the expression of glial fibrillary acidic protein (*GFAP*), oxytocin receptor (*OXTR*), and tyrosine hydroxylase (*TH*) in the brain.

*Keywords*: fluorescence *in situ* hybridization, fluorescence methods, *Gasterosteus aculeatus*, gene expression, gene localization, *in situ* hybridization, RNA, threespine stickleback.

## INTRODUCTION

Threespine stickleback are renowned for their phenotypic variation. An increasing number of studies are identifying genes related to morphological (Shapiro *et al.*, 2004; Colosimo *et al.*, 2005; Miller *et al.*, 2007; Liu *et al.*, 2014), hormonal (Kitano *et al.*, 2010), and behavioural (Greenwood *et al.*, 2013) differences between stickleback populations. Behavioural studies are also identifying genes whose expression is influenced by the social environment in sticklebacks (Sanogo *et al.*, 2011, 2012; Rittschof *et al.*, 2014; Greenwood and Peichel, 2015).

Linking genes to traits requires additional techniques to determine both how and where gene expression changes within a tissue. Subtle changes in the location of gene expression

Correspondence: N. James, Neuroscience Program, University of Illinois at Urbana-Champaign, 505 South Goodwin Avenue, Urbana, IL 61801, USA. email: nholmes3@life.illinois.edu
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can have major consequences for trait development. For instance, pelvic reduction in freshwater sticklebacks is due to the loss of *Pitx1* expression specifically in the developing pelvic girdle but not in other tissues (Shapiro *et al.*, 2004; Chan *et al.*, 2010). qPCR is useful for validating how expression levels differ, but its resolution is limited to the precision of the dissection. Alternatively, antibody staining can be used for localization of proteins, but developing new antibodies can be expensive and time-consuming.

For fine localization of gene expression, a visualization technique (Tautz and Pfeifle, 1989) such as fluorescence *in situ* hybridization (FISH) is necessary. In this technique, a labelled RNA probe binds its complementary mRNA, expressed from the targeted gene. FISH is not limited to mRNA; it can be used to label any type of expressed RNA including microRNAs and long non-coding RNA (lncRNA). FISH has superb resolution, allowing for subcellular localization (Zimmerman *et al.*, 2013) and the possibility of detecting the expression of multiple genes simultaneously using different fluorescent labels (Barroso-Chinea *et al.*, 2007). FISH can also be used to distinguish qualitative differences in expression between upregulation in the same cells and new expression in previously silent cells. Therefore, we optimized a protocol for FISH on sectioned stickleback tissue. Although we established the protocol for brain tissue, we note key steps where it can be optimized for other tissues.

## PROTOCOL

The fish used in the development of this protocol were wild-caught adult threespine stickleback (male and female) collected from Putah Creek, California, USA. All procedures received approval from the University of Illinois at Urbana-Champaign Institutional Animal Care and Use Committee (protocol #15077). Product ordering information together with specific primers for our probes are listed in their entirety in the 'Materials' section following the protocol. All steps are carried out at room temperature unless otherwise specified.

The FISH protocol comprises six distinct phases, as outlined in Fig. 1. The general strategy is to label the target mRNA, which requires a specific probe. As tissue penetration (Kühn and Köster, 2010) can hamper deep tissue labelling, thin sections are necessary. We compare FISH on two dissection techniques (i.e. flash-frozen tissue vs. fresh tissue) and on two embedding techniques (i.e. cryo- vs. paraffin embedding). We also show the expression of glial fibrillary acidic protein (*GFAP*), oxytocin receptor (*OXTR*), and tyrosine hydroxylase (*TH*) in the brain as representative results.

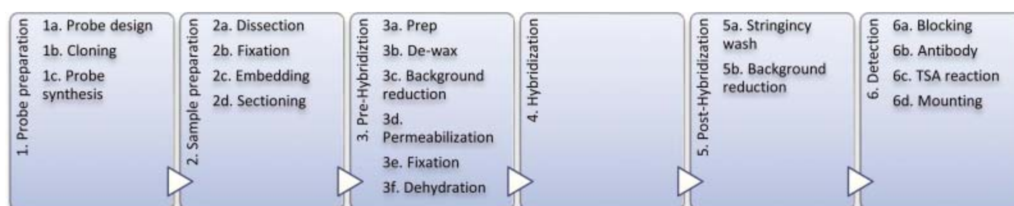


**Fig. 1.** Workflow for FISH broken into the six major phases of the protocol. Note that Phase 2 takes a minimum of 2 days, while Phases 3 and 4 should be done on the same day.

**Important:** Use RNase-free solutions and tools unless otherwise noted. Degradation of either the RNA probe or the mRNA target will result in weak or no signal.

## 1. Probe preparation

### 1a. Probe Design

Design a probe that recognizes more than one exon, preferably with 50% GC content. Spanning an intron with the probe reduces the background that can occur from genomic labelling. Alternatively, probes can be designed to the 3′UTR (Thisse and Thisse, 2008) to reduce cross-reactivity via a unique sequence. The anti-sense strand of the target sequence will be used as the probe. For a negative control, use the sense strand, a scramble sequence, or a gene not expressed in the relevant tissue. This negative control will be used to check for non-specific labelling. Always check to ensure the uniqueness of a probe by comparing potential target sequences to the stickleback genome assembly (found on the UCSC Genome Browser) using the BLAT feature.

### 1b. Cloning

Using whole brain (or tissue of interest) cDNA collected from a non-experimental fish, do a nested PCR (in which the inner primer set amplifies a region within the product of the outer primers) reaction to amplify the gene's target sequence. The presence of a single band at the expected size by gel electrophoresis should confirm specificity of primers. Transform the checked PCR product into a vector (pCRII) with both T7 and SP6 promoters using a TOPO® TA Cloning Kit. Sequence the inserts to confirm the identity of the probe and to determine the correct sense transcription start site. Alternatively, SP6 and T7 promoter sites can be added to the inner PCR primers to avoid the need for vector transformation. We used both methods of probe design during the testing of this protocol.

### 1c. Probe Synthesis

Template DNA should be cleaned, concentrated, and resuspended in RNase-free water. It can be generated from either nested PCR or amplified from a long-term storage clone/library so long as it has appropriate RNA polymerase start sites. The probe synthesis reactions follow the Roche DIG RNA Labelling Kit (SP6/T7). After a 15–30 minute DNase I treatment, ethanol precipitate the probe with lithium chloride for at least 1 hour at −80°C and resuspend in aliquots of 50 $ng \cdot \mu L^{-1}$ in RNase-free water for storage at −20°C.

## 2. Sample preparation

### 2a. Dissection

Decapitate an experimental fish. For the flash-freezing protocol, immediately immerse the head in a dry ice-ethanol bath for 5 minutes until the eyes turn cloudy white. Next, for either protocol, in a room temperature environment, remove the brain from the skull using RNase-free (cleaned with RNaseZap) tools.

We compared FISH on flash-frozen or fresh brain tissue and did not observe an effect on either morphology (Fig. 2a) or FISH signal (Fig. 2b) regardless of embedding method. Flash-freezing the head can ease dissection by preventing complications from accidental damage to surrounding tissue (e.g. eye punctures), but makes the brain more liable to chipping from the Micro-Rongeurs during extraction. Ultimately, both techniques are viable.

### 2b. Fixation

Fix the brains immediately following dissection for 12–24 hours at 4°C in RNase-free 4% paraformaldehyde (PFA). The 4% PFA (pH 7.4 in PBS) should be <2 months old and stored in individual aliquots to avoid freeze/thawing. For paraffin embedding, a standard overnight fixation (12–18 hours) is sufficient. However, we found that a longer fixation of 20–24 hours was necessary for cryo-embedding.

### 2c. Embedding

We compared FISH on cryo- and paraffin-embedded tissue. While both produced specific labelling (Fig. 3b), paraffin better preserved the tissue morphology (Fig. 3a). Cryo-sectioning has less processing time and lower initial equipment costs. However, paraffin embedding reliably allows thinner (<10 $\mu$m) sectioning, resulting in more sections from a given brain. With paraffin embedding, the concerns are that heat and harsh (toluene) chemicals might damage mRNAs. However, we did not observe signal degradation with our probes. RNA was stable in both paraffin blocks (stored at −20°C) and slide-mounted sections (at room temperature for testing; we recommend storage at −20°C) for more than 1 month. Cryo-embedded samples were stored in blocks (at −20°C) until ready for sectioning and immediate processing.

*Paraffin embedding*: Immediately following fixation, transfer tissue to RNase-free 70% ethanol (EtOH). To begin the embedding process, move the sample to an appropriately sized mesh tissue cassette; for example, for brain, we used a Micromesh Biopsy cassette. Run the tissue, either automatically using a Tissue-Tek VIP or by hand, through the following series of washes:

- Equilibrate in an EtOH gradient of 70%, 80%, 95%, 95%, 100%, 100%, 100% for 40 minutes each.
- Wash in 50% toluene/EtOH for 40 minutes.
- Wash twice in 100% toluene for 1 hour each.
- Wash three times in paraffin at 60°C for 40 minutes each.

Hold the samples in the final paraffin wash at 60°C before embedding in a paraffin block using an embedding station. The Tissue-Tek base mould 7 × 7 × 5 mm fit adult brains best, minimizing excess paraffin.

*Cryo-embedding*: Fill the cryo-mould with room temperature tissue freezing media. We tested use of a sucrose gradient prior to media immersion but found no improvement to tissue preservation, so the brain may be transferred directly from the fixative. Add the fixed tissue and push to the bottom, oriented such that the desired sectioning plane (sagittal, transverse or coronal) is parallel to the bottom. Place the mould on dry ice and allow the block to freeze completely. The media turns white when fully frozen.

### 2d. Sectioning

Tissue damage is most likely to occur during sectioning. Uneven or torn sections indicate that the blade was not sharp enough. For cryo-embedded samples, torn tissue can also indicate sectioning was done at an improper temperature or humidity for the tissue type. Wrinkling and folding of the tissue arises when the embedding material curls. We

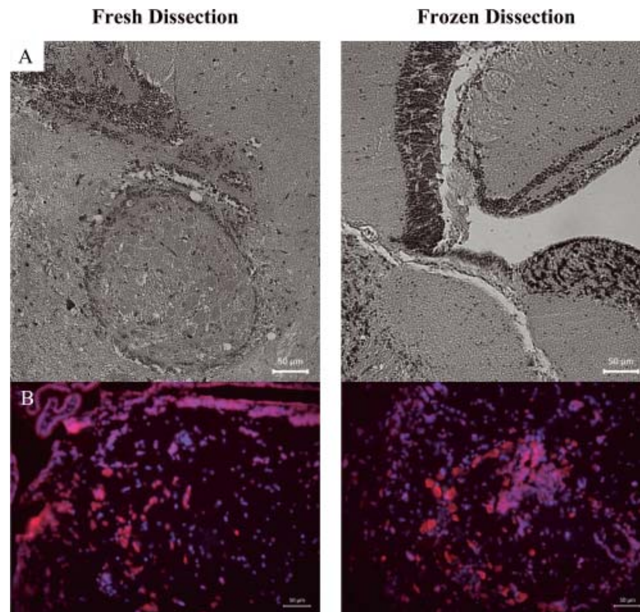


**Fig. 2.** Following decapitation, the brain was either immediately placed in fixative or the head was flash-frozen in ethanol on dry ice prior to brain extraction. There was no notable difference in either (A) morphology preservation or (B) signal quality. Tissue was treated simultaneously and identically following dissection. Paraffin-embedded transverse sections of (A) the diencephalon with H&E (haemotoxylin and eosin) staining, and (B) the posterior telencephalon with the probe *PAC1b-R* (red) and Hoechst nuclear dye (blue). The images were taken sequentially with no light adjustment between the images.

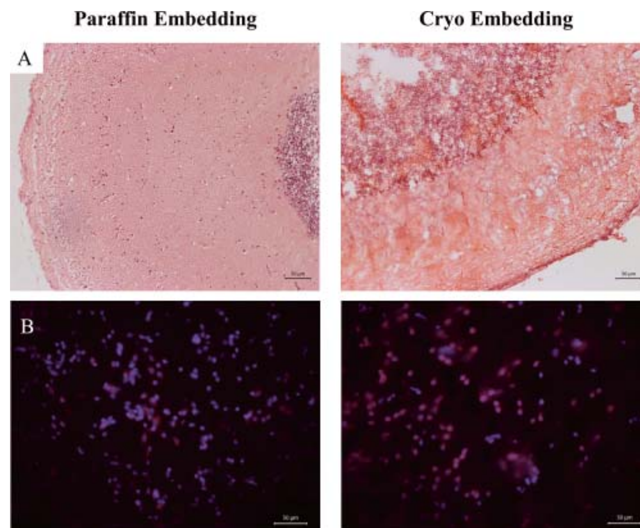


**Fig. 3.** We compared FISH on paraffin- and cryo-embedded tissue. (A) Paraffin embedding resulted in better morphology preservation. (B) Signal quality was consistent between embedding methods. Tissue was fresh dissected and processed for FISH simultaneously. Sections of the (A) tectum opticum with H&E staining and (B) mid telencephalon with the probe *PAC1b-R* (red) and Hoechst nuclear dye (blue).

apportioned serial sections across 8–16 slides for adult brain tissue, resulting in a series of sections at a variety of depths on each slide.

*Paraffin*: Section (8–10 $\mu$m) on a microtome. Immediately float sections on 15% ethanol and then on 42°C water before affixing them to a slide. These 30-second to 5-minute floats smooth wrinkles in the tissue and help the section bind to the slide. Dry the slides on the slide heater for >24 hours before proceeding with labelling.

*Cryo*: Section (10–25 $\mu$m) on a cryostat within 24 hours of embedding. Allow sections to thaw for 5 minutes at room temperature, adhering them to the slide before either storing at –20°C or proceeding with labelling.

## 3. Pre-hybridization

### 3a. Preparation

3a(1): Calculate temperatures for hybridization ($T_{hyb}$) and stringency washes ($T_{wash}$). Generally, probes with similar $T_{hyb}$ can be processed in batches to minimize the need for multiple hybridization ovens or runs. If, for example, there are three genes at $T_{hyb}$ of 57/58/59°C, it is usually sufficient to hybridize them all at 58°C.

| $T_{hyb} = T_m - 25$ | $T_{wash} = T_m - 14$ |
|---|---|
| The $T_m$ is calculated by Wilkinson's formula for RNA : RNA in solution:<br><br>$T_m = 79.8 + 18.5\log[Na] + 58.4(GC\ fraction) + 11.8(GC\ fraction)^2 - (820/\text{Probe length (bp)}) - 0.35(formamide\%)$<br><br>Calculate $T_m$ using 60% formamide, 5× saline sodium citrate (SSC) buffer (Na = 0.825) | |

3a(2): Pour 1× Target Retrieval Solution into plastic Coplin jars (glass jars will break) and heat in a 95–100°C bath. This solution will be used in 3c(3) ('Background reduction').

**Note:** For cryo-sections, rinse slides in RNase-free $H_2O$ for 3 minutes, then skip to 3c 'Background reduction'.

### 3b. De-wax

3b(1): Preheat slide heater to 55–60°C.
3b(2): Melt wax from tissues in slide heater for 15 minutes and immediately proceed, in a Coplin jar, with washes.
3b(3): Wash three times in Clearene for 5 minutes each.
3b(4): Wash three times in 100% EtOH for 5 minutes each.
3b(5): Wash in 90% EtOH for 5 minutes.
3b(6): Wash in 70% EtOH for 5 minutes.
3b(7): Wash in RNase-free $H_2O$ for 5 minutes.
3b(8): Remove any remnant Clearene or alcohol in the water. This is typically only a problem if slides were placed back-to-back in Coplin jars.

### 3c. Background Reduction

Acetylation with triethylamine (TEA) and acetic anhydride prevents non-specific binding of the probe, thereby reducing background signal. This chemical reaction substitutes an amine group ($–NH–CO–CH_3$) in place of the reactive amine group ($NH_3^+$). Antigen retrieval combats the deleterious effects of formaldehyde fixation and paraffin embedding. Antigen retrieval is especially important for genes that are expressed at a low level because it increases the signal-to-noise ratio. TSA-based detection (covered in step 6c) requires quenching of endogenous peroxidase activity, which we accomplish through incubation in hydrogen peroxide ($H_2O_2$) with methanol.

3c(1): Move slides to TEA solution (2 mL 2 M TEA per 40 mL RNase-free $H_2O$) in a Coplin jar and add 100 $\mu$L of acetic anhydride per 40 mL of TEA solution. Cap, invert to mix, and place on shaker for 10 minutes.
3c(2): Rinse in RNase-free $H_2O$ for 5 minutes.
3c(3): Transfer slides to the preheated (95–100°C) Target Retrieval Solution. Heat for 15 minutes with the cap slightly loosened.
3c(4): Take Coplin jar out of water bath and allow it to cool for 25–30 minutes on a shaker.
3c(5): After jar cools, add RNase-free 1× PBS and allow to sit for 5 minutes.
3c(6): Switch slides to 3% $H_2O_2$ (4 mL of 30% $H_2O_2$ diluted in 36 mL methanol to make 40 mL, made fresh from < 2-month-old stock) for 30 minutes up to 2.5 hours.
3c(7): Move to fresh Coplin jar with RNase-free 1× PBS and allow to shake for 5 minutes. If the slides are placed back-to-back, transfer one-by-one so that remnant methanol is rinsed off the slides. Pull out proteinase K to thaw upon starting 5-minute shake.
3c(8): Rinse once more with RNase-free 1× PBS for 5 minutes.

### 3d. Permeabilization

Proteinase K treatment allows the probe better access to the target by partially eliminating proteins, especially those associated with nucleic acids. This is a critical step of the ISH protocol according to the *DIG Application Manual for Nonradioactive In Situ Hybridization* (Eisel *et al.*, 2008) that will need to be optimized for different tissues. Both time and concentration can be varied to achieve optimum probe penetration with the least tissue damage.

3d(1): Make 5 $\mu g \cdot mL^{-1}$ proteinase K solution in new RNase-free 1× PBS in a Coplin jar. Add slides to the solution and allow to sit for 10 minutes.

### 3e. Fixation

Additional on-slide fixation is important for stabilizing tissue and nucleic acids after permeabilization. It can also improve the adhesion of sections to slides, reducing sample damage or loss.

3e(1): Fix in RNase-free 4% paraformaldehyde (made from 13 mL 12% stock diluted in 39 mL RNase-free 1× PBS) for 10 minutes.
3e(2): Wash in RNase-free 1× PBS and allow to shake for 5 minutes. If slides are back-to-back, separate and return to jar so that remnant PFA is washed out.
3e(3): Wash again in RNase-free 1× PBS for 5 minutes.
3e(4): Wash in glycine (made fresh, 3.7 g in 40 mL of RNase-free 1× PBS) for 20 minutes. After starting the wash, preheat the hybridization oven and solution to $T_{\mathbf{hyb}}$ calculated in step 3a(1).
3e(5): Wash in RNase-free $H_2O$ for 5 minutes.

3f. Dehydration

Dehydrating the sections prior to hybridization prevents dilution of the probe and hybridization buffer and helps affix tissues to the slide. However, this can also result in increased non-specific labelling, especially on the edges of tissues. Dilute all solutions with RNase-free water and use repeatedly for up to 2 months.

3f(1): Rinse in 50% EtOH for 2 minutes.
3f(2): Rinse in 70% EtOH for 2 minutes.
3f(3): Rinse in 90% EtOH for 2 minutes.
3f(4): Rinse in 100% EtOH for 2 minutes.
3f(5): Rinse in 100% EtOH for 2 minutes.
3f(6): Allow slides to dry completely on a clean kimwipe for 5–10 minutes. While slides are drying, place 6–20 $\mu$L of RNA probe (225–375 ng) in 250 $\mu$L of hybridization solution and heat for 5 minutes in a heat block at 80°C.

## 4. Hybridization

The recommended hybridization buffer contains 60% formamide and has a sodium ion ($Na^+$) concentration of 0.825 moles (from 5× SSC). Alternatively, ULTRAhyb Ultrasensitive Hybridization Buffer can be used with a 50% formamide concentration, but the $T_m$ calculation used in step 3a(1) will need to be adjusted. There are many formulations of hybridization buffers; we did not test alternative mixtures. To facilitate binding, probes need to be denatured (linearized) via pre-heating before being placed on the samples.

4a: Place 6–20 $\mu$L of RNA probe (225–375 ng) in 250 $\mu$L of hybridization solution and heat for 5 minutes in a heat block at 80°C. This can be done while the slides are drying (3f(6)).
4b: Place heated probe/hyb mix on ice to cool while loading slides.
4c: Add probe/hyb mix to slides, being careful to avoid bubbles. Pipette probe solution lengthwise onto the top edge of a plastic hybri-slip (remove plastic covers from both sides), then lower one long edge of the slide down onto the cover slip, so that the solution wicks up onto the tissue and the hybri-slip sticks onto the slide. Alternatively, pipette the probe mix directly onto the samples and slowly lower the hybri-slip.
4d: Seal the hybri-slip in place using rubber cement along the edges and place in a humidified chamber. Hybridize overnight at $T_{hyb}$.

## 5. Post-hybridization

5a. Stringency Wash

Post-hybridization, stringency washing eliminates non-specific hybridization, thereby boosting the signal-to-noise ratio. It also removes any excess unbound probe.

5a(1): Set water bath to $T_{wash}$ calculated in step 3a(1) and pre-warm the four stringency solutions.
5a(2): Take slides out of the hybridization chamber and remove hybri-slips by prying them off with a pair of forceps. If a cover is stuck because the cement fully dried, a 1-minute soak in 5× SSC should soften the cement.
5a(3): Place slides in a glass Coplin jar with 5× SSC for 10 minutes at room temperature.

**Important:** From this point forward, do not let the tissue dry out completely.

5a(4): Place slides in pre-warmed 5× SSC and place in a water bath for 10 minutes.
5a(5): Wash in pre-warmed Wash I (5× SSC/50% formamide) for 20 minutes.
5a(6): Wash in pre-warmed Wash II (0.5× SSC/50% formamide) for 20 minutes.
5a(7): Wash in pre-warmed 0.5× SSC for 10 minutes.
5a(8): Wash at room temperature in 0.5× SSC for 10 minutes.

5b. Background Reduction

At this point, the mRNA and probe are bound and inaccessible to RNases. Iodoacetamide treatment reduces non-specific antibody binding by reacting with disulphide bridges and sulphydryl groups (Allen Institute for Brain Science, Technical White Paper).

**Note:** Use deionized water from this step forward for solutions; RNase-free water is no longer necessary.

5b(1): Place slides in 20 mM iodoacetamide (0.148 g in 40 mL water) for 5 minutes.
5b(2): Rinse in 1× Tris-buffered saline, with Tween (TBST) for 10 minutes.

## 6. Detection

6a. Blocking

6a(1): Remove slide from Coplin jar, dry back and edges and draw a rectangle with wax pen around tissue to contain the antibody solution. Add 100–200 $\mu$L of TNB block buffer.
6a(2): Incubate for 30 minutes at room temperature. Make sure the slides are level so that the sections don't dry out. Place in a closed box to reduce evaporation.

6b. Antibody

Using a separate nuclear label is preferable to a mounting medium combined with nuclear label (e.g. Vectashield with DAPI), as the intensity of the nuclear dye can be adjusted.

6b(1): With a sharp flick of the wrist, shake off the TNB and add 100–200 $\mu$L of Anti-Dig-POD diluted 1:300 in TNB. Optionally, add a nuclear stain to the antibody mix. We recommend using Hoechst at 1 $\mu$L (10 $\mu$g) for up to 1 mL of solution.
6b(2): Incubate for 1 hour at room temperature in a closed box.

**Important:** Check halfway to ensure that slides do not dry out. Add more antibody mix as required.

6b(3): Wash slides in a Coplin jar three times in 1× TBST for 5 minutes.

6c. TSA Reaction

Probe signal is enhanced using Tyramide Signal Amplification (TSA) for better detection of genes expressed at low levels. The final deionized water washes remove ions that cause a blue glow under fluorescent light sources.

6c(1): Pull out slides from TBST 2–3 at a time and dry the back and edges as before while avoiding the wax outline. Quickly add 100 $\mu$L of TSA with rhodamine, diluted 1 : 100 in 1× amplification buffer provided in the kit. Incubate for 20–30 minutes.

Alternatively, follow the manufacturer's recommendation of a 1 : 50 dilution with a 6-minute incubation.

6c(2): Wash slides in a Coplin jar three times in TBST for 5 minutes.

6c(3): Wash slides twice for 5 minutes in deionized water.

6d. MOUNTING

Before mounting, you can check the signal quality of both the TSA reaction and the nuclear staining by covering the samples with water and a coverslip. If additional staining is needed, remove the coverslip, wash in TBST and repeat the necessary reaction (Hoechst or TSA) followed by the final washes. While Vectashield is used in this protocol, any anti-fading medium can be used, including Prolong Gold.

6d(1): Lay the glass cover slips on the counter and place a thin smear of mounting media (Vector H-1000) lengthwise on the top edge of each slip. Take out the slides from the deionized water, dry back and edge as before, making sure any excess water has dripped off. Lower the slides onto the cover slip and allow the media solution to wick along the tissue without any air bubbles. If there are air bubbles on top of the tissue, remove them by gently lifting and replacing the coverslip.

6d(2): Store slides protected from light. Scan slides as soon as possible, since weak signals will fade.

## MATERIALS

| Item name | Manufacturer | Item # |
|---|---|---|
| TOPO® TA Cloning Kit, Dual Promoter | Invitrogen | 45-0640 |
| Roche DIG RNA Labelling Kit (SP6/T7) | Roche | 11175025910 |
| RNaseZap | Ambion | AM9780 |
| Micromesh Biopsy cassette | Simport | M507-2 |
| Tissue-Tek base mould (7 × 7 × 5mm) | EMS | 4161 |
| Tissue-Tek Cryomold | EMS | 4566 |
| Tissue freezing media | Leica | 14020108926 |
| Slides | *For paraffin sectioning*:<br>Leica Surgipath X-tra<br>*For cryo-sectioning*:<br>Fisher Superfrost Plus | <br>38002052<br><br>12-550-15 |
| 10 × Target Retrieval Solution | 10 mM sodium citrate, 0.05% Tween 20, pH 6.0 | For 1 litre, 2.96 g tri-sodium citrate (dehydrate), 1 N HCl, 0.5 mL Tween |

| Item name | Manufacturer | Item # |
|---|---|---|
| Clearene | Leica Surgipath | 3803600 |
| TEA (triethanolamine) | Sigma | T1377 |
| Acetic anhydride | Sigma | A6404 |
| 30% Hydrogen peroxide | Sigma | H1009-100 mL |
| Proteinase K solution (PCR grade) | Roche or any other company | 03115828001 |
| mRNA *in situ* hybridization buffer | See recipe below<br>*Or*: Ambion ULTRAhyb | <br>AM8669 |
| Hybri-slips (plastic) 24×20 mm | Sigma | H1034 |
| Iodoacetamide | Sigma | I1149-25g |
| Formamide | Sigma | F7508 |
| TNB block buffer | See recipe below | Need blocking reagent #FP1020 Perkin Elmer |
| TSA with rhodamine (tetramethyl rhodamine tyramide) | Perkin Elmer | NEL742B001KT (250–750 slides total)* |
| 20× SSC | 0.15 M NaCl, 0.015 M sodium citrate | For 1 litre, 175.3 g NaCl, 88.2 g sodium citrate |
| Vectashield mounting media | Vector Labs | H1000 |
| Hoechst | Molecular Probes | H3570 |
| 10× TBST | DAKO or make below | S3306 |
| Wax Pen (Super Pap Pen) | Invitrogen | 00-8899 |
| Anti-Digoxigenin-POD | Roche | 11-207-733-910 |

* TSA kit: This kit has 10 vials of tyramide with rhodamine. Dilute the solid tyramide in each vial in this kit with 150 μL of DMSO.

| TNB block buffer | 10 × TBST | Hyb buffer |
|---|---|---|
| 0.1 M Tris-HCl, pH 7.5 | 0.5 M Tris-HCL, pH 7.5 | 12 mL 20× SSC |
| 0.15 M NaCl | 3 M NaCl | 30 mL deionized formamide |
| 0.5% blocking reagent from | 1% Tween 20 | 12 mL dextran sulphate |
| Perkin Elmer FP1020 | Dilute 1:10 to getting working solution of TBST | 300 μL Denhardt'ssolution |
| | | 900 μL yeast tRNA |
| | | 1200 μL salmon sperm DNA |

| Gene and Ensembl ID | Primers |
|---|---|
| Adenylate cyclase activating polypeptide 1b (pituitary) receptor type I (*PAC1b-R*)<br>ENSGACG00000005402 | *Outer*:<br>5′-CATGTCCCGGAGACACAAGT-3′<br>5′-TTGTCCTGCATGTAGCGGAT-3′<br>*Inner* (for cloning):<br>5′-ATTCAGTGACGTGGAACCCG-3′<br>5′-TTTGAGCCTCGAACCCGATG-3′<br>*Inner* (PCR method):<br>5′-ATTTAGGTGACACTATAGATTCAGTGACGTGGAACCCG-3′<br>5′-TAATACGACTCACTATAGGGTTTGAGCCTCGAACCCGATG-3′ |
| Glial fibrillary acidic protein (*GFAP*)<br>ENSGACG00000009804 | *Outer*:<br>5′-CGAATTGGCAGCCTTTCTTCC-3′<br>5′-GGGCTCCTTCCCCTTAAACT-3′<br>*Inner* (for cloning):<br>5′-GATCACCTTGGGCTCAACCA-3′<br>5′-GTTCTGCTGCTCCAAAAGGC-3′<br>*Inner* (PCR method):<br>5′-ATTTAGGTGACACTATAGGATCACCTTGGGCTCAACCA-3′<br>5′-TAATACGACTCACTATAGGGGTTCTGCTGCTCCAAAAGGC-3′ |
| Oxytocin receptor (*OXTR*)<br>ENSGACG00000000914 | 5′-CGAACCCTCTCAAGCGGAAT-3′<br>5′-ACAGCATGTCTGGTCCGTAG-3′ |
| Tyrosine hydroxylase (*TH*)<br>ENSGACG00000011104 | 5′-AGTGAATACCTCGTGGCCCT-3′<br>5′-GGAGTGACGGATGTACTGGG-3′ |

## EQUIPMENT

| Item name | Manufacturer | Item # |
|---|---|---|
| Embedding station | Leica | EG1150 H |
| Tissue-Tek VIP | Sakura | 3000 |
| Microtome | Leica | RM2255 |
| Cryostat | Leica | CM1850 |
| Slide heater | TBS | SD-11-120 |

## REPRESENTATIVE RESULTS

We further tested the protocol using several different probes on paraffin-embedded adult female stickleback brain tissue, as this was our preferred embedding method. The FISH protocol was sensitive, as we detected signal with concentrations as low as 1 $\mu g \cdot mL^{-1}$, and used 5–10 $\mu g \cdot mL^{-1}$ for most probes. Conserved neuronal markers were synthesized into probes and a sample expression pattern for an astrocyte marker (GFAP) is shown in Fig. 4a.

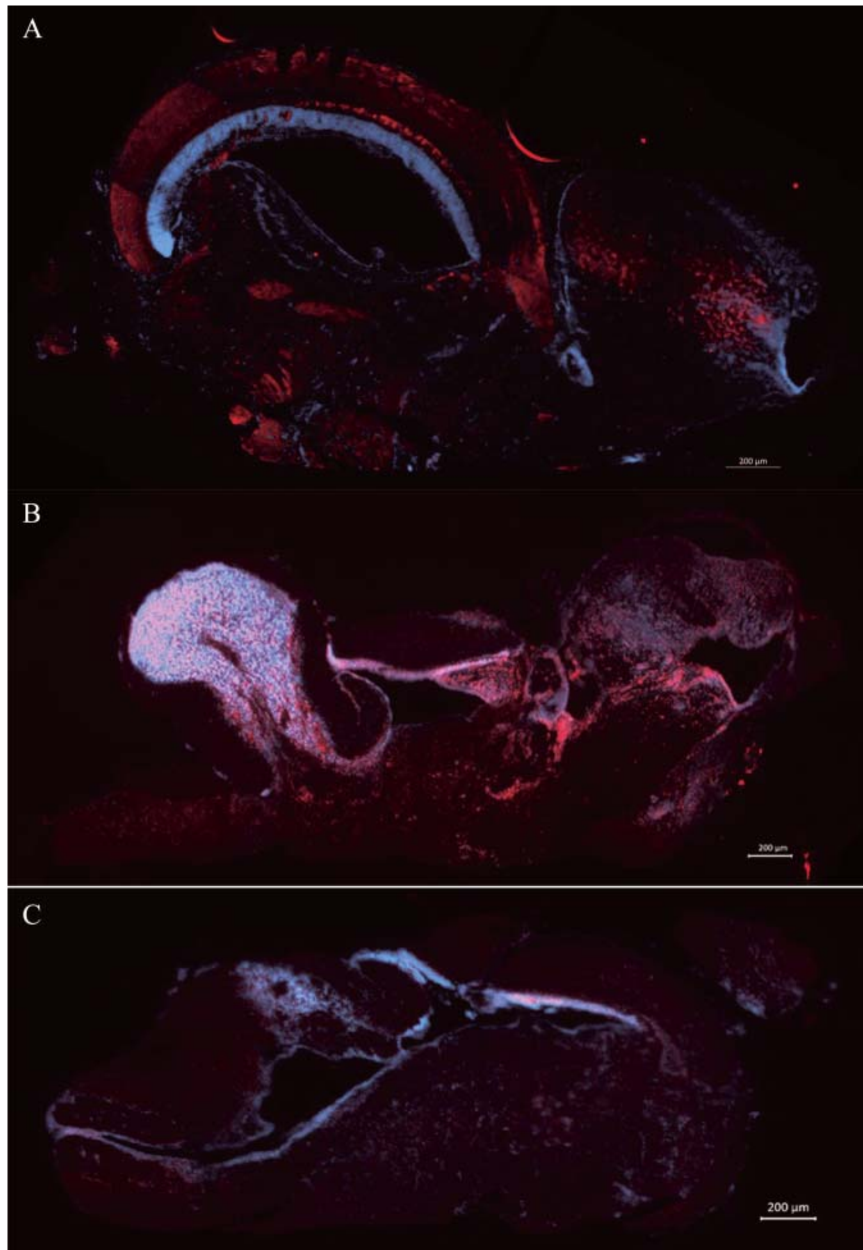


**Fig. 4.** Representative results as visualized on a Zeiss Axiovert 200M. Sagittal sections with the anterior facing right and the dorsal oriented towards the top. The gene of interest is labelled in red and nuclei are blue. (A) Glial fibrillary acidic protein (*GFAP*) is a marker of astrocytes. (B) Oxytocin receptor (encoded by *OXTR*) is the receptor for the neuropeptide oxytocin. (C) Tyrosine hydroxylase (encoded by *TH*) is the rate-limiting enzyme in the synthesis of catecholamines.

The expression patterns of select genes relevant to social behaviour including oxytocin receptor (*OXTR*) and tyrosine hydroxylase (*TH*) are shown in Fig. 4b and 4c respectively.

## DISCUSSION

We established a protocol for fluorescent *in situ* hybridization (FISH) to localize gene expression on sectioned stickleback brain tissue. Brain dissection technique (fresh or frozen) did not affect morphology or final signal. Both cryo- and paraffin embedding resulted in successful FISH signal throughout the brain. The cryo-embedding process was faster, taking 4 days from dissection to labelled slides, compared with a minimum of 5 days with paraffin embedding. However, paraffin embedding resulted in better tissue preservation and thinner sections.

An increasing number of studies (Shapiro *et al.*, 2004; Colosimo *et al.*, 2005; Miller *et al.*, 2007; Kitano *et al.*, 2010; Greenwood *et al.*, 2013; Liu *et al.*, 2014) have identified QTL or genes relevant to ecologically important traits in sticklebacks. Neurogenomic studies have identified hundreds of genes whose expression levels are influenced by the biotic environment, including the social environment (Sanogo *et al.*, 2011, 2012; Rittschof *et al.*, 2014; Greenwood and Peichel, 2015). By allowing us to visualize gene expression patterns in complex tissue, FISH will help stickleback biologists attempting to mechanistically link genes to phenotypes.

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