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Comparative *in situ* hybridization protocols in zebrafish

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ABSTRACT

In situ hybridization is a commonly used technique in molecular biology to assess the temporal and spatial expression of a given gene. As a long and labor-intensive protocol, double *in situ* hybridization, which detects two genes in series, is challenging and can require a lot of troubleshooting. Optional additives, polyvinyl alcohol and dextran sulfate, were tested in a standard *in situ* hybridization protocol and several colorimetric stain pairings using double *in situ* hybridization in zebrafish embryos. Optional additives can improve staining time and reduce nonspecific background. Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (BCIP) + Fast Red/BCIP was the most effective stain pairing. As a proof-of-concept, this work shows that *Cabin1* and *atoh1b* are expressed in distinct regions of the developing zebrafish brain.

METHODS SUMMARY

A comparison of colorimetric stains and protocols in double *in situ* hybridization in whole-mount zebrafish embryos.

KEYWORDS:

atoh1b ● *Cabin1* ● comparative ● gene expression analysis ● protocol ● stain ● technique ● zebrafish

In situ hybridization (ISH) is used to assess gene expression patterns in tissues. It is commonly used in many species in both single and double forms. There are many different stains and technique variations used, leading to confusion. The major advantage of using colorimetric (also called chromogenic) stains over the more sensitive fluorescent signals [1] is that alkaline phosphatase (AP) colorimetric reactions can be easily monitored in real-time for signal intensity and background. Fluorescent methods can be cost-prohibitive and have been assessed in zebrafish by others [1–4]. Nitro-blue tetrazolium chloride (NBT) paired with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) produces an indigo precipitate with a relatively strong signal and low background, making it the most commonly used substrate. Colorimetric counterstains can vary greatly in sensitivity [5]. Volume exclusion agents are optional reagents in some protocols. Polymers like dextran sulfate and polyvinyl alcohol (PVA) are intended to maximize reactions by taking up solvent space and locally concentrating reactants to reduce stain times and nonspecific background stain [6–8].

Double colorimetric ISH is valuable because it is a modification of widely used protocols, using techniques and reagents that are readily available. Given the goal of this technique to visualize gene expression patterns at an organism or tissue level, the ability to compare potentially overlapping gene expression patterns of two genes in the same sample offers more information than comparing embryo or tissue samples. The current work was designed to evaluate the potential benefits of two optional additives and compare common stains in double ISH in zebrafish to identify a pair of stains that could detect overlapping signals. The gene expression profiles of *atoh1b* and *Cabin1* were compared individually with different stains and additives and together in double ISH.

Materials & methods

Animals

Zebrafish husbandry and experimental procedures were approved by the Institutional Animal Care and Use Committee. Adult fish from the AB strain (ZIRC, OR, USA) were housed in recirculating rack systems (Aquaneering, CA, USA) at 27.5°C on a 14:10 light:dark cycle and fed twice daily with Zeigler Adult Zebrafish Complete Diet (Pentair, MN, USA). Embryos were generated by natural spawning, fixed in 4% paraformaldehyde and stored at -20°C in methanol.

Probe synthesis

Primers were designed to isolate probe templates for *Cabin1* (5′-AGTAAAGGCCGAGTGCTGAA-3′ and 5′-CACTTACTGCGCTCTGA-3′) [9] and *atoh1b* (5′-CTGAGCACGGCATTCTTTAT-3′ and 5′-TCCTCCAGTGTGTCCTTCTTC-3′). The resulting templates were ligated into a pGEM T Easy Vector System (Promega, WI, USA), transformed into chemically competent DH5α *E. coli*, purified and sequenced. Digoxigenin (DIG) and fluorescein (FLU)-labeled riboprobes were generated from PCR amplified templates [10] using primers that span the T7 and SP6 RNA polymerase binding sites of the pGEM-T Easy Vector (forward: 5′-GACCATGATTACGCCAAGC-3′; reverse: 5′-

GGCGATTAAGTTGGGTAACG-3′). Sense and antisense probes were generated for each gene. The probe synthesis reaction contained a final concentration of 250 ng of purified PCR product, 1 mM adenosine triphosphate, 1 mM cytidine triphosphate, 1 mM guanosine triphosphate, 0.65 mM uridine triphosphate, 0.35 mM DIG-11-UTP or FLU-11-UTP (Roche Applied Sciences, IN, USA), 40 U RNase OUT (Invitrogen, MA, USA), 0.4 U thermostable inorganic pyrophosphatase (New England Biolabs, MA, USA), 20 U T7 or SP6 phage RNA polymerase, 1 × transcription buffer and 10 mM dithiothreitol (DTT; Promega). Template DNA was destroyed with 20 U RNase-free DNase (Roche). Probes were precipitated in 100% ethanol with nuclease-free sodium acetate to a final concentration of 115 mM, then pelleted, washed in ice-cold nuclease-free 70% ethanol, dried and resuspended in nuclease-free water with 2–3 U/μl RNase OUT. The probes were analyzed via NanoDrop spectrophotometry (Thermo Fisher Scientific, IL, USA) , nondenaturing gel electrophoresis and an abbreviated *in situ* dot blot protocol conducted by crosslinking probes to a positively charged nylon membrane (Supplementary Figure 1).

Single *in situ* hybridization

The single ISH protocol used to detect the expression patterns of single genes was a modification of Thisse *et al.* [11]. Embryos were rehydrated through a series of methanol + 1 × phosphate-buffered saline (pH 7.4) + 0.1% Tween20 (PBTween) washes before being digested for 5 min in 10 μg/ml proteinase K, fixed for 20 min in 4% paraformaldehyde and washed again in PBTween. Embryos were incubated overnight at 65°C in DIG-labeled probes in a prehybridization solution (50% formamide, 1.5 × saline-sodium citrate (SSC), 5 μg/ml heparin, 9.25 mM citric acid, 0.1% Tween20 and 50 μg/ml yeast tRNA; Invitrogen, MA, USA). Embryos were washed at 75°C in a series of increasing stringency steps, blocked in 5% normal sheep serum + 2% bovine serum albumin + 1% dimethylsulfoxide in PBTween and then incubated in 1:5000 sheep AP-conjugated anti-DIG Fab fragments (Roche Applied Sciences, IN, USA) in blocking solution overnight at 4°C. The excess antibody was washed away with PBTween before the embryos were equilibrated in a buffer of 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5 and 0.1% Tween20 (NTMT buffer). Embryos were stained in culture plates in 4.5 μl/ml NBT and 3.5 μl/ml BCIP in NTMT buffer in the dark. Staining was allowed to proceed until background just began to appear in the sense controls unless otherwise noted. A detailed protocol is available at dx.doi.org/10.17504/protocols.io.j8nlkk33wl5r/v1.

Pigmentation

Two common methods to reduce the interference of embryo pigmentation were compared with pigmented embryos. To prevent pigmentation, embryos were incubated in and treated with 0.2 mM 1-phenyl-2-thiourea (PTU) in 30% Danieau beginning at gastrulation. To reduce pigment after fixation, embryos were bleached in 3% H_2O_2 and 1.79 mM KOH for 5 min. Except where noted, all experiments use bleached embryos.

Permeabilization

The standard method to increase the permeability of embryos is to briefly digest embryos in 10 μg/ml proteinase K in PBTween following rehydration steps. Digestion lasted 5 min; embryos were then fixed in 4% paraformaldehyde for 20 min at room temperature and washed in PBTween before hybridization. This method was compared with another method that uses 80% acetone/20% water at room temperature for 20 min and followed by washing in PBTween before hybridization [12].

Volume exclusion agents

PVA was added at a final concentration of 10% to the NTMT buffer in an altered protocol: 1 ml 1 M Tris pH 9.5, 200 μl 5 M NaCl and 8.8 ml water were heated to 90°C and then allowed to cool to 60°C. PVA was added slowly and shaken while incubating in a water bath at 60°C. Once in solution, the mixture was cooled to room temperature. The remaining 500 μl 1 M $MgCl_2$ and 50 μl 20% Tween20 were then added to the buffer mixture just before the NBT and BCIP stains. Dextran sulfate (Alfa Aesar, MA, USA) was added to the prehybridization and hybridization solutions to a concentration of 5% [2]. Embryos were otherwise treated as previously described.

Double *in situ* hybridization

Double ISH detects two genes through serial staining [13]. Embryos were rehydrated, digested in proteinase K, fixed in 4% paraformaldehyde and incubated in prehybridization (Prehybe) buffer as in single ISH. Embryos were incubated overnight in both probes. Volume-excluding agents were not used. Embryos were washed in a series of increasing stringency solutions. Embryos were then blocked and incubated overnight in their first antibody (Table 1). The excess antibody was washed off and the first probe was stained. The excess from the first stain was washed off and the embryos were incubated in 0.1 M glycine HCl pH 2.2 to remove the first antibody [11]. Following PBTween washes, embryos were incubated in a second antibody and stained.

Staining

Several counter stains were tested. Unless otherwise noted, NBT/BCIP was used as the second stain and embryos were always stained in culture plates in the dark. To counterstain NBT/BCIP with Fast Red (Sigma Aldrich, MO, USA), which generates a red precipitate, embryos were first incubated with anti-FLU diluted in a block and second with 1:2000 anti-DIG antibody. Fast Red stain and buffer tablets were dissolved in 1 ml water and sonicated. The use of diaminobenzidine (DAB; Thermo Fisher Scientific, IL, USA) substrate, which generates a brown precipitate, requires a peroxidase (POD)-conjugated antibody and a series of steps to block endogenous PODs

Table 1. Stain and antibody pairings.

Stain	Antibody	Antibody concentration	Color	Stain time in dISH
NBT/BCIP	AP-conjugated anti-DIG or anti-FLU Fab fragments	1:5000 or 1:2000	Purple	2–4.5 h
Fast Red	AP-conjugated anti-FLU Fab fragments	1:2000	Red	2–3 days
Vector Red	AP-conjugated anti-FLU Fab fragments	1:2000	Red	Not detected
DAB	POD-conjugated anti-FLU Fab fragments	1:200	Brown	5 h
Fast Red/BCIP	AP-conjugated anti-DIG or anti-FLU Fab fragments	1:2000 or 1:1000	Cyan	2–4 days

AP: Alkaline phosphatase; BCIP: 5-Bromo-4-chloro-3-indolyl phosphate; DAB: Diaminobenzidine; DIG: Digoxigenin; dISH: Double *in situ* hybridization; FLU: Fluorescein; NBT: Nitro-blue tetrazolium chloride; POD: Peroxidase.

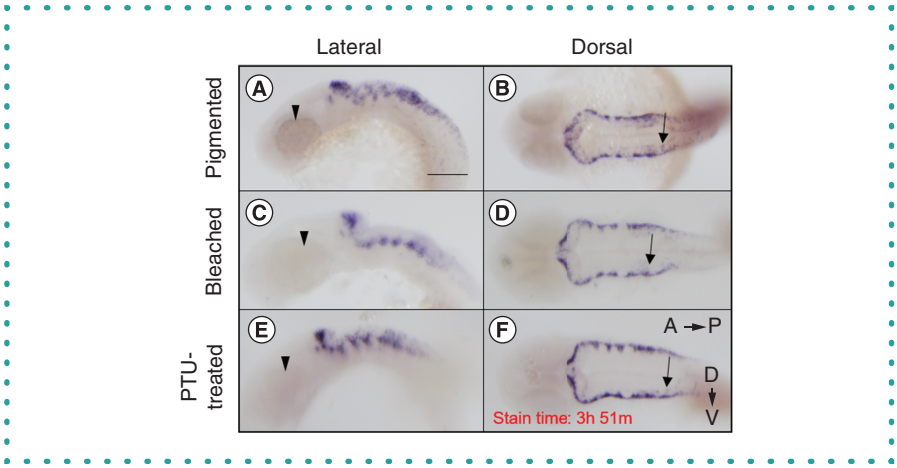

Figure 1. Bleaching reduces superficial signal detection. (A & B) First natural pigmentation is visible in the developing retina (arrowhead) at 24 h postfertilization. Untreated embryos have the clearest, most detailed *atoh1b* staining in the hindbrain (arrow). **(C & D)** Postfixation bleaching in H_2O_2 eliminates pigmentation in the retina (arrowhead) but reduces expression detail in the hindbrain (arrow). **(E & F)** Treatment with 0.2 mM PTU prevents pigmentation in the retina (arrowhead) with minimal reductions in staining clarity in the hindbrain (arrow). Scale bar = 100 μm.
PTU: 1-phenyl-2-thiourea.

were incorporated after the posthybridization washes. Embryos were incubated for 5 min in 50% MeOH/PBTween, 10 min in 100% MeOH, 20 min in MeOH + 0.3% H_2O_2 and 5 min in 50% MeOH/PBTween and then rinsed with PBTween. The antibodies used were 1:200 sheep POD-conjugated anti-FLU Fab fragments (Roche Applied Sciences, IN, USA) and 1:5000 anti-DIG. Embryos were transferred from their final PBTween wash to the DAB substrate diluted to 1× in DAB buffer solution, according to the manufacturer's instructions (Thermo Fisher Scientific, IL, USA). To counterstain with Vector Red (Vector Laboratories, CA, USA), which produces a red precipitate, embryos were equilibrated in 0.1 M Tris HCl (pH 8.2) + 0.1% Tween and then stained according to the manufacturer's instructions. To counterstain Fast Red + BCIP (FR/BCIP), which yields a bright cyan precipitate [14], with NBT/BCIP, Fast Red was prepared fresh as previously described. Then, 5.6 μl/ml Fast Red and 3.5 μl/ml BCIP were added to the NTMT buffer. Detailed protocols are included in the Supplementary Materials and at dx.doi.org/10.17504/protocols.io.4r3l2onnxv1y/v1.

Imaging

Embryos were fixed in 4% paraformaldehyde, rinsed and suspended in glycerol. Some embryos were manually deyolked. Images were captured with a Nikon SMZ1270 DS Fi3 (Nikon USA, NY, USA) with accompanying software and cropped, color balanced and assembled into montages using Photoshop CC (Adobe, CA, USA).

Results & discussion

Methods to improve embryo clarity can affect signal intensity

As natural pigmentation can obscure stain detection, researchers commonly bleach embryos postfixation or chemically prevent pigmentation in live embryos before fixation. The signal detection in untreated, H_2O_2 bleached and PTU-treated embryos were compared. Pigmentation is visible in the developing retina at 24 h postfertilization (hpf; Figure 1A & B). Bleaching eliminates retinal pigmentation and reduces detection sensitivity (Figure 1C & D). PTU treatment prevents retinal pigmentation with minimal disruption of signal sensitivity (Figure 1E & F).

PTU treatment effectively blocks the synthesis of pigments, although the concern among researchers is the ability of developmental exposure to this chemical to alter gene expression patterns [15–17]. Bleaching embryos has thus become an industry standard because

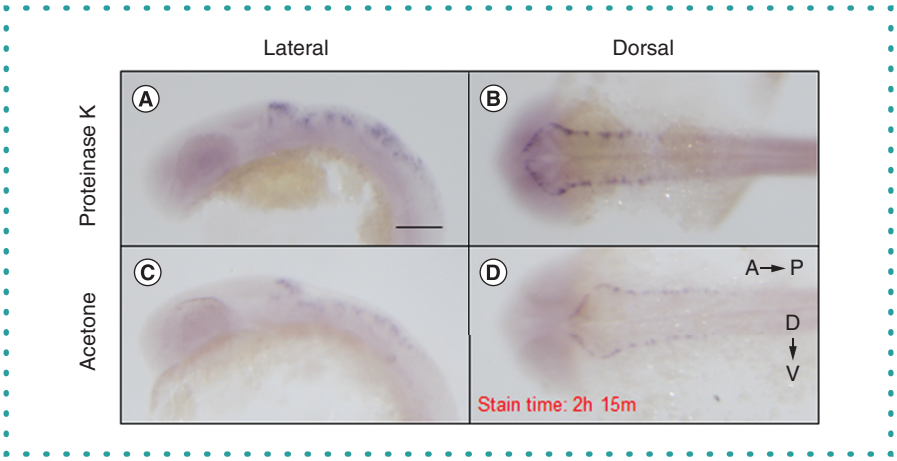

Figure 2. Comparing permeabilizing agents. Expression at 24 h postfertilization with **(A & B)** standard 10 μg/ml proteinase K treatment versus **(C & D)** 80% acetone permeabilization. Embryos treated with proteinase K stained more rapidly. Embryos were deyolked for clarity. Embryos were naturally pigmented. Scale bar = 100 μm.

it does not affect gene expression, as the animals are already fixed. Thus, the current studies all used bleached embryos. Mutant strains of fish have been used to generate pigment-free embryos, which can eliminate the harsh effects of these chemical methods of reducing pigmentation [18,19]. However, using these embryos requires maintaining additional fish lines, which are no longer wildtype fish, adding expense, labor and potential confounds. Alternatively, if pigmentation does not interfere with the desired gene expression pattern being detected due to the developmental stage or expression pattern, the current results suggest that the use of pigmented embryos is superior.

Methods to permeabilize tissue

Proteinase K is widely used to permeabilize tissue for ISH, although other methods are also used. Proteinase K and acetone permeabilization were compared when detecting *atoh1b* at 24 hpf (Figure 2). When allowed to stain side by side for the same duration (just over 2 h) proteinase K-treated embryos (Figure 2A & B) exhibited much more signal intensity than acetone-treated embryos (Figure 2C & D). Proteinase K-treated embryos also exhibit much higher background signal.

Two protocols for permeabilizing embryos were compared, although other methods also exist, such as trypsin-mediated permeabilization [12]. For a gene that is expressed superficially, like *atoh1b*, the use of acetone permeabilization offered no benefit and would require more staining time to reach the same intensity level as proteinase K-treated embryos. However, the main advantage of using acetone is likely in the detection of deep signals without sacrificing tissue quality necessitated by prolonged proteinase K treatment in these thicker tissues [12].

Additives can reduce staining time & improve signal clarity

Without any additives, NBT/BCIP detection of *atoh1b* using DIG-labeled probes takes approximately 4 h. *Atoh1b* expression first appears after approximately 2 h (Figure 3A & D). At this same time, the addition of PVA or dextran sulfate yields more intense staining (Figure 3). When staining continues to completion (the point at which sense controls begin to show color), which took on average 3–4 h, *atoh1b* is clearly defined with slight background stain near the eyes (Figure 3G & J). *Atoh1b* stains granule cell precursors in the upper rhombic lip and lateral hindbrain at 24 hpf, consistent with previous reports [20]. The signal in these regions is intense and discrete. The addition of PVA increased the intensity of the signal (Figure 3H & K) but did not reduce the background. The addition of dextran sulfate to the prehybridization and hybridization solutions intensified the signal and reduced the background, revealing additional detail in the hindbrain (Figure 3I & L).

Volume-excluding additives can improve signal detection [2,7]. While PVA was expected to reduce staining time, the incorporation of PVA increased the amount of labor invested in the protocol. Others have reported that PVA incorporation can improve the signal to noise in long incubations [21], although the present results suggest that PVA decreases the staining time without necessarily reducing the background. The addition of dextran sulfate to the prehybridization and hybridization steps increased the strength of staining in genes and allowed the detection of regions previously too weak for discrete staining, which is consistent with previous reports [2,21]. Dextran sulfate is inexpensive and nonhazardous, is easy to incorporate into the hybridization solution, shortens staining times and improves the signal to nonspecific background stain.

Single *in situ* hybridization alternative stains

Stains in single ISH were compared to determine which gene would get which stain compared with NBT/BCIP as the standard (Figure 4). *Atoh1b* is expressed in a smaller spatial area at a higher intensity than *Cabin1*. *Atoh1b* is expressed in the rhombic lip and lateral hindbrain.

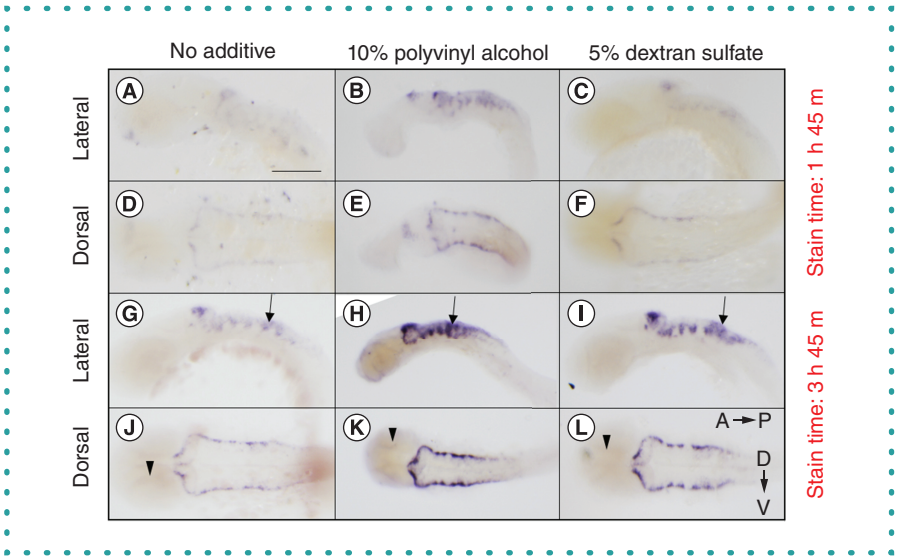

Figure 3. Additives improve signal detection. Nitro-blue tetrazolium chloride paired with 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) staining of *atoh1b* compared with and without volume excluding additives in 24 h postfertilization zebrafish embryos. **(A–F)** Embryos from the same batch were stained until first color appearance in controls **(A & D)**. **(G–L)** Embryos stained to completion. **(B, E, H & K)** Polyvinyl alcohol (10%) enhances signal intensity of real and background stain (eye region; arrowheads). **(C, F, I & L)** Dextran sulfate (5%) increases sensitivity (hindbrain; arrow) without increasing background. Embryos were manually deyolked. Scale bar = 100 μm.

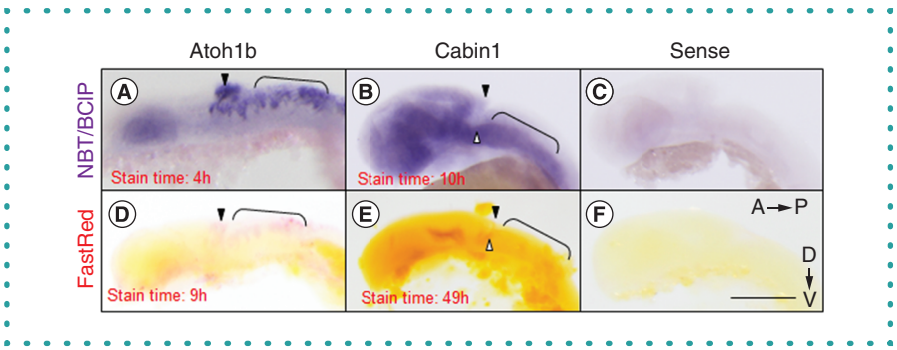

Figure 4. Single *in situ* hybridization for *atoh1b* demonstrates the superiority of NBT/BCIP as a stain. (A–C) NBT/BCIP is more sensitive and offers higher contrast. **(D–F)** Fast Red displays less nonspecific staining in red, although discolors the otherwise clear embryo yellow. **(A & D)** *Atoh1b* is expressed in the rhombic lip (filled arrowhead) and hindbrain (bracket) in zebrafish embryos at 24 h postfertilization. Both stains label the predicted region and display paler, nonspecific staining, particularly in the eye. **(B & E)** *Cabin1* is expressed in many brain regions, including the ventral rhombic lip (open arrowheads) and hindbrain (bracket). **(C & F)** Sense probes for *Cabin1* served as controls. Embryos were manually deyolked. Scale bar = 100 μm. NBT/BCIP: Nitro-blue tetrazolium chloride paired with 5-bromo-4-chloro-3-indolyl phosphate.

Cabin1 is expressed in several parts of the brain, including the rhombic lip and hindbrain. Fast Red reveals similar *atoh1b* patterns of staining and background as NBT/BCIP, with an overall lower intensity, poorer signal to background and longer stain time (9 h vs 4 h). Fast Red detection of *Cabin1* was restricted to the most robust expression areas (Figure 4E) and much slower (49 h vs 10 h).

Double *in situ* hybridization

Because of its reliability, NBT/BCIP was used in conjunction with Fast Red, DAB, Vector Red and Fast Red + BCIP. Staining times vary depending on several conditions, such as sequence abundance, probe concentration and quality, antibody titer and fixation time. As such, stain times are reported in relation to the single stain time using standard NBT/BCIP in addition to the raw time (Table 1). When *Cabin1* was stained with NBT/BCIP, it masked the *atoh1b* signal (Figure 5A). Therefore, *Cabin1* was detected with the alternative stain and *atoh1b* with NBT/BCIP. Fast Red took approximately six times longer to stain than standard NBT/BCIP. (Figure 5B). DAB stained embryos typically in half the time of NBT/BCIP, but background expression in whole-mount preparations was high. This renders the true DAB signal weak in dual-stained embryos (Figure 5C). When co-labeling with Vector Red, NBT/BCIP signal must be detected first, per the manufacturer's protocol. Subsequent Vector Red stain was never red in our experiments. Embryos were uniformly yellowish and pale after 20 min and darkened uniformly yellowish with increased staining time (hours). This dark yellowish stain did not change over 4 days,

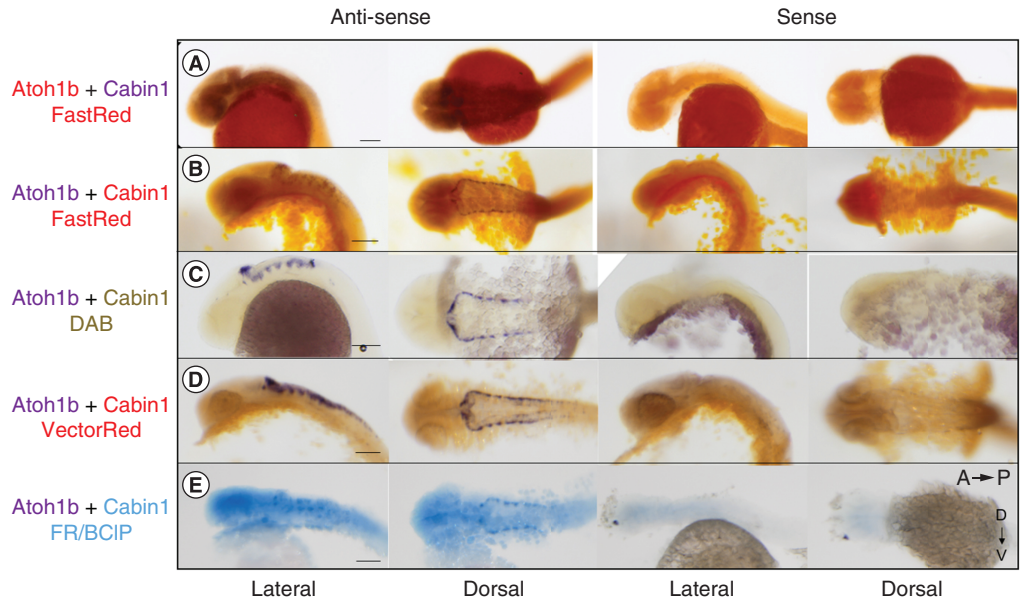

Figure 5. Stain pairings in double *in situ* hybridization vary widely in efficacy. Lateral and dorsal views of each gene pair with sense probes for comparison following antisense probes. **(A)** *Cabin1* detected with NBT/BCIP (indigo) obscures *atoh1b* detected with Fast Red (red). **(B–E)** *Atoh1b* detected with NBT/BCIP (indigo). **(B–D)** Fast Red (red), DAB (brown), and Vector Red (yellow) are not sensitive enough to detect *Cabin1*. **(E)** Fast Red/BCIP labels *Cabin1* without masking *atoh1b*. Sense probes do not reveal specific staining. Embryos were manually deyolked. Scale bars = 100 μm. DAB: Diaminobenzidine; NBT/BCIP: Nitro-blue tetrazolium chloride paired with 5-bromo-4-chloro-3-indolyl phosphate.

after which we determined no real signal was to be detected (Figure 5D). Vector Red stain did not significantly disrupt the first stain in the double ISH trials. Fast Red paired with BCIP (FR/BCIP) improved the signal intensity in diffuse *Cabin1* staining. FR/BCIP took, on average, ten times longer than standard NBT/BCIP staining (49 h). The dark NBT/BCIP indigo remained distinct against cyan FR/BCIP (Figure 5E).

NBT/BCIP is the most common colorimetric stain because of its superior sensitivity and high contrast. Except where noted, NBT/BCIP was used as the second stain, as the long procedure can reduce the signal intensity of the second gene [1]. NBT/BCIP can mask the less intense color of the second stain, so the most sensitive stain was used last. Staining with the weaker stain first enabled monitoring of the appearance of an additional signal, even if it ultimately masked the original signal later. This is in contrast to other protocols that recommend detecting the fainter probe signal with NBT/BCIP [1]. The present results instead support detection of the weaker probe signal first, as the protocol reduces the signal intensity of subsequent probes, and then layering the more intense signal second. One caveat is the relative expression patterns of the two sequences. It was easier to detect the widespread and diffuse *Cabin1* expression with the weaker signal since we could let it stain for a very long time without the risk of masking the second signal. The more discrete and intense signal from *atoh1b* detected by any of the less intense stains would be masked by diffuse *Cabin1* expression detected by NBT/BCIP. The interchangeability of the stains and AP-conjugated antibodies makes it easy to switch the order of stains.

Fast Red can be an effective although less sensitive stain in single ISH. Fast Red can be effective in differentiating nonoverlapping intense expression patterns because the red and indigo colors are easy to distinguish [1,22,23]. However, when the goal is to detect overlapping or diffuse signals, such as those detected here, as a colorimetric stain, Fast Red was not useful in double ISH because the signal was faint and so easily masked by NBT/BCIP. However, Fast Red does fluoresce, which may reveal weak or masked signals [22]. DAB staining uses a POD-conjugated antibody, which does not risk interference with the AP-conjugated antibody used to detect the other gene. NBT shows through DAB staining more easily than other stains, which is useful for colocalization, provided the DAB signal is sufficiently robust to allow detection above the high background associated with DAB staining. Given the high degree of background in the present experiments, DAB is of low value in whole-embryo hybridizations. The bright cyan precipitate generated by FR/BCIP stains embryos slowly but with low background. When detected first, FR/BCIP can be tracked even when signals from NBT/BCIP overlap. This stain incorporates the higher sensitivity of BCIP paired with a different AP substrate, Fast Red, to generate a precipitate that is sufficiently distinct from the NBT/BCIP indigo. This allows better detection of lower abundance transcripts. These results corroborate reports using chick embryos of FR/BCIP as an effective counterstain [14]. These counterstains represent a nonexhaustive list of commonly used alternatives to NBT/BCIP. We did not test iodonitrotetrazolium because of its low sensitivity [22], nor did we test alternatives to NBT/BCIP, such as BM Purple [12], which stains darker than even NBT/BCIP. In some cases, double chromogenic ISH still may not be successful,

such as when detecting two low abundance transcripts or when background levels are consistently high. In such scenarios, the high sensitivity of fluorescent ISH would likely offer better results. One limitation of double ISH is that the series of harsh treatments and extensive washes can damage embryos and thus repetition is necessary to confirm results.

We compared *atoh1b* and *Cabin1* expression in the developing zebrafish brain, particularly in the cerebellum anlage and hindbrain to determine if *Cabin1* is expressed in *atoh1b*-positive granule cell precursors. The zebrafish cerebellum develops from two progenitor zones in the anterior hindbrain: the rhombic lip and the ventricular zone. The upper rhombic lip progenitors express *atoh1* and produce glutamatergic granule neurons [24]. *Atoh1b* is found in the very posterior upper rhombic lip and slightly dorsal lateral hindbrain, consistent with previous reports [20]. *Cabin1* is localized to the deeper ventral and medial parts of the rhombic lip and hindbrain. *Cabin1* has no known paralogs and is a conserved, calcium-dependent repressor protein [25–28]. Its function in the developing brain is unknown. The current findings suggest *Cabin1* is excluded from granule cell precursors.

Conclusion

From these experiments described here, three conclusions can be drawn. First, the addition of 5% dextran sulfate to the hybridization solution is a low-cost, easy-to-implement optional additive that yields the marked advantages of clearer signal, reduced background discoloration and shorter staining times. Second, Fast Red/BCIP was the most effective counterstain to NBT/BCIP in double ISH in 24 hpf whole zebrafish embryos. Finally, the experiments demonstrated that *Cabin1* and *atoh1b* are both expressed in nonoverlapping regions of the developing zebrafish cerebellum and hindbrain at 24 hpf.

Future perspective

ISH is widely used in molecular biology to discover gene expression patterns and evaluate their changes following perturbation [21]. Even as transgenic reporter lines allow fluorescent, highly sensitive detection of gene expression patterns, the validation of these lines relies on ISH [29]. Therefore, ISH remains a valuable gene expression technique. The advent of additional stains, both colorimetric and fluorescent, has expanded the usefulness of ISH to coincidently detect two or more sequences. Zebrafish continue to grow in popularity as a model organism, making the comparison and validation of these ongoing modifications essential to such a widely used technique.

Summary points

- The aim of the current work was to compare variations in protocol and colorimetric stains in double *in situ* hybridization in zebrafish embryos.

Results

- The inclusion of 5% dextran sulfate in the hybridization solution reduced stain time and improved signal detection.
- Fast Red/5-bromo-4-chloro-3-indolyl phosphate (BCIP; cyan) paired with nitro-blue tetrazolium chloride/BCIP (indigo) is the most effective combination for detecting overlapping or diffuse gene expression patterns in colorimetric double *in situ* hybridization in zebrafish embryos.
- *Cabin1* and *atoh1b* are expressed in distinct parts of the zebrafish hindbrain.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2022-0038

Author contributions

DR Hammond-Weinberger and K Dunn designed the experiments and wrote the manuscript; all authors contributed to conducting the experiments, analyzing the data and approving the content of the manuscript.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

Zebrafish husbandry and all experimental procedures were approved by the Murray State Institutional Animal Care and Use Committee (IACUC).

Data sharing statement

All data are included in this manuscript.

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