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Multiplex Fluorescent *In Situ* Hybridization in Zebrafish Embryos Using Tyramide Signal Amplification

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ABSTRACT

One of the strengths of the zebrafish is the ease with which *in situ* hybridization can be performed to determine spatial and temporal patterns of gene expression in whole embryos. Thus far, colorimetric detection methods are mainly used for these analyses. Here we describe a fluorescent *in situ* hybridization (FISH) protocol for whole-mount zebrafish embryos using tyramide signal amplification (TSA). An optimal set of reagents was identified that allows for simultaneous localization of gene expression patterns of two genes within the same embryo, permitting identification of colocalized expression within single cells. This protocol can be extended to perform multiplex studies by repetition of the TSA-based detection for each target sequentially with a different fluorescent dye label. To this effect, we demonstrate that this approach can be combined with standard horseradish peroxidase (HRP)-mediated immunocytochemistry procedures in addition to FISH.

INTRODUCTION

Of the many important tools available for use in zebrafish, whole-mount *in situ* hybridization (WISH) has proved to be especially useful for temporal and spatial resolution of gene expression because of the optical transparency of the embryos and early swimming larvae.[1,2] Using hybridization of labeled antisense RNA, the localization of specific transcripts can be visualized in whole embryos, providing positional information about the expression of a gene of interest at specific developmental stages. The currently used colorimetric detection methods are not amenable to multiparameter assays, however, seriously limiting the potential of the technique for multigene assays. Available methods depend on precipitation of chromogenic substrates such as BCIP/NBT or DAB, catalyzed by either alkaline phosphatase (AP) or horseradish peroxidase (HRP), respectively,[3] and are prone to generating high background and diffusion artifacts.[4] Chromogenic substrates can be used simultaneously to localize spatially distinct transcripts in a single embryo but are not useful for discerning overlapping or colocalized gene expression patterns because they are difficult to discriminate visually when deposited at the same site.

Fluorescent *in situ* hybridization (FISH) has the potential to be a powerful strategy to overcome the limitations of colorimetric-based detection schemes and visualize multiple gene-specific transcripts in the same embryo and their colocalization in single cells.[5] Two-color FISH protocols have been described in the zebrafish using the fluorescent alkaline phos-

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phatase substrates Fast Red and ELF-97, which provide red and green fluorescence, respectively, but are limited by their low sensitivity.[3] In addition, very few fluorescent alkaline phosphatase substrates are commercially available, inherently limiting the application of this approach to multiplex studies.

Ideally, zebrafish FISH protocols should have high sensitivity and resolution, low background, and be able to utilize a number of different fluorescent channels in order to maximize the number of signals that can be imaged simultaneously. Optimized multiplex FISH protocols with these characteristics are available for *Drosophila*,[5] providing for visualization of seven distinct gene expressions patterns simultaneously. The most sensitive method of fluorescent mRNA detection uses tyramide signal amplification (TSA).[6] Fluorescent TSA utilizes the enzymatic activity of HRP and fluorescent dye-tyramide substrates, generating highly reactive tyramide species that become covalently deposited at the site of enzyme activity. The availability of this technology with bright, water-soluble Alexa Fluor® dye tyramides (Molecular Probes, Eugene, OR) provides for sensitive, high-resolution detection ranging from ultraviolet (UV) to far-red. Application of FISH technology to the zebrafish, however, has been problematic likely because the comparatively large size of the embryo, and high background resulting from autofluorescence has limited its sensitivity.

Here, we describe an optimized protocol for fluorescent TSA-based FISH in zebrafish embryos. Importantly, we demonstrate that the detection protocol can be performed repeatedly on the same specimen using a different fluorescent label for each gene target; the HRP-mediated detection is performed for each target sequentially by inactivating the HRP after each labeling and applying a new HRP conjugate to detect the next target with a different fluorescent dye-tyramide conjugate. We tested a variety of reagent combinations and determined an optimal set of reagents that provide high signal-to-noise ratios. In addition, we show that this multiplex FISH procedure can be combined with standard HRP-mediated immunocytochemistry protocols, thereby maximizing the information that can be obtained from single embryos.

RESULTS

Selection of Reagents

The TSA protocol initially created a very specific background staining pattern present in all tested fluorescent channels in the absence of RNA probe, which was dependent upon the BSA in blocking buffer (Fig. 1). The absence of blocking buffer led to high ubiquitous background but use of a traditional blocking buffer (2 mg/mL BSA, Sigma A-3294) produced a consistent background signal most prominent

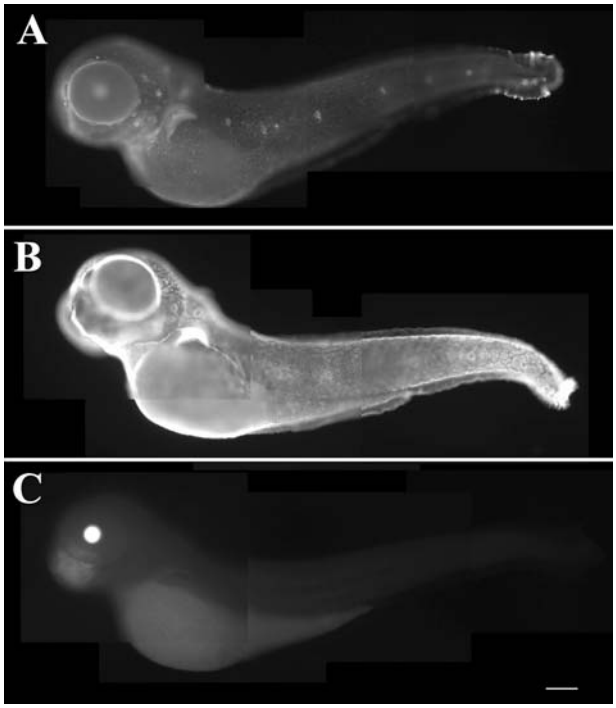

FIG. 1. Fluorescent images of background signal detected with FISH protocol in the absence of probe, using reagents for digoxigenin (DIG) staining on 4 days postfertilization (dpf) embryos. Pictures are shown for the red fluorescent channel but are also characteristic of the background in the green, ultraviolet, and cyan channels. (**A**) Background signal detected using traditional bovine serum albumin (BSA)-based blocking buffered described in the experimental procedures. (**B**) Background signal detected in the absence of blocking buffer. (**C**) Background signal using Roche Western blocking reagent described in Materials and Methods. (**A**) and (**C**) are taken with 0.5-second exposure, (**B**) is taken with 0.05-second exposure to avoid light saturation. Scale bar, 100 μm.

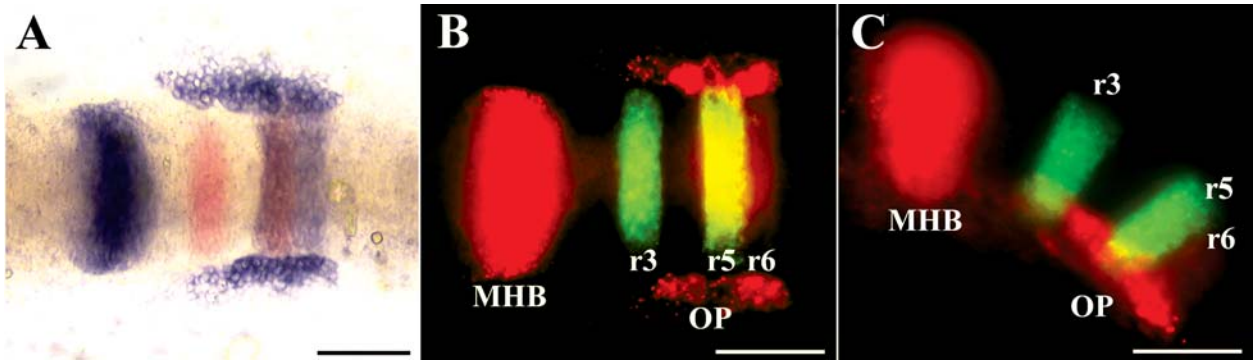

FIG. 2. *In situ* hybridization using *pax2a*-DIG, *val*-DIG, and *egr2b*-fluorescein. (**A**) AP color detection of DIG-labeled probes (purple) and fluorescein-labeled probes (pink). (**B**) and (**C**) Fluorescent overlay images of tyramide signal amplification (TSA) detection of DIG-labeled probes (red) and fluorescein-labeled probes (green); colocalization appears yellow. (**A**) and (**B**) are flatmount preparations of 5–10 somite embryos, (**C**) is left lateral view of dorsal aspect. MHB, mid-hindbrain boundary; r, rhombomere; OP, otic placode. Scale bars, 100 μm.

in the neuromasts, and along the fin (Fig. 1A,B). The use of a Western blocking reagent (Roche 1921673) that consists of 10% casein protein in maleic acid buffer eliminated essentially all background signal (Fig. 1C). In addition, some of the primary antihapten antibodies we tested also produced background staining in the absence of probe. Of the primary reagents tested, we achieved the best signal-to-noise ratio using the sheep derived primary antibodies from Roche. The use of a secondary linked to HRP rather than a primary helps to further amplify the signal prior to TSA visualization of weaker probes. In order to eliminate cross reactions between secondary antibody and the two sheep derived primaries, a high concentration (1:200) of secondary HRP-conjugated anti-sheep was used to saturate the signal from the initial primary prior to HRP inactivation and deposition of the following primary anti-

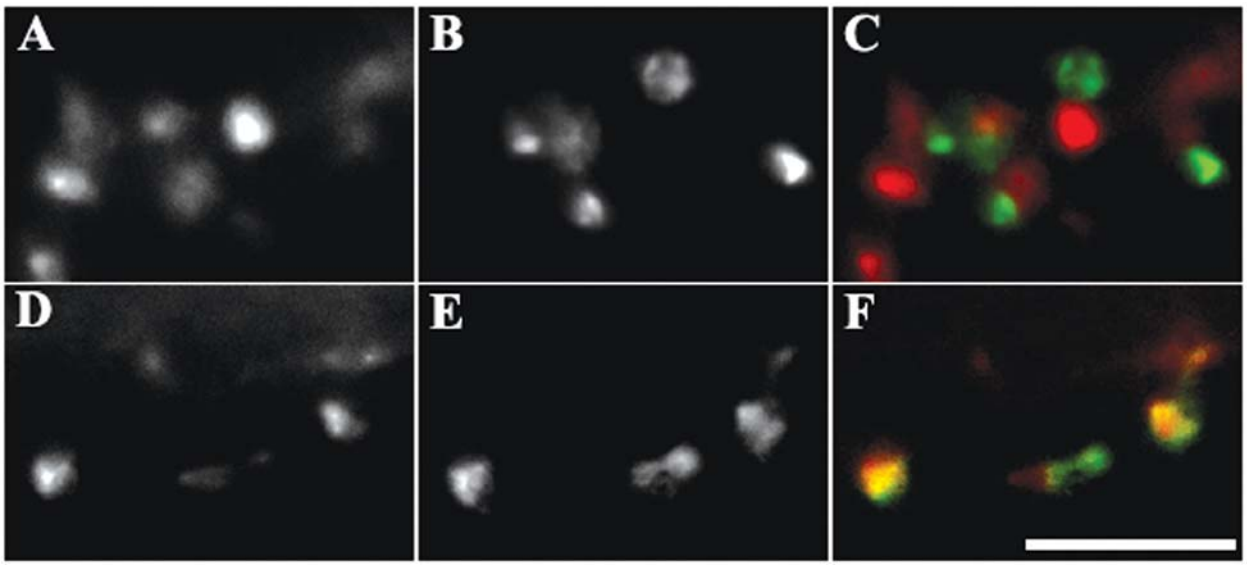

FIG. 3. Double fluorescent *in situ* hybridization (FISH) of whole-mount zebrafish embryos. (**A–C**) Fluorescent images of 1 day postfertilization (dpf) embryo caudal vein imaged for *fms*-DIG antisense RNA visualized with red fluorescence (**A**) and *mpo*-fluorescein antisense RNA visualized with green fluorescence (**B**), and overlay (**C**). (**D–F**) Fluorescent overlay of 1 dpf embryo caudal vein imaged for *fms*-DIG antisense RNA visualized with red fluorescence (**D**), and *l-plastin*–fluorescein antisense RNA visualized with green fluorescence (**E**), and overlay (**F**); colocalization appears yellow. Scale bar, 50 μm.

body. All TSA detection was done with the Alexa Fluor® conjugates. We found the Alexa Fluor® series were stable, gave bright signal, and were easily separated using standard filters for the fluorescent microscope. Using TSA detection, signals from strong or moderately strong probes can be readily identified during the 30-minute to 1-hour detection time required for the TSA reaction. For weaker probes, the primary antibody concentration can be increased up to 20-fold without increasing background.

The Alexa Fluor® conjugates are available in a variety of fluorescent channels. We have tested fluors for 4 channels: red (Alexa Fluor® 555), green (Alexa Fluor® 488), cyan (Alexa Fluor® 410) and UV (Alexa Fluor® 310). Older embryos (> 2 dpf) have almost no red autofluorescence, weak green autofluorescence, intermediate cyan autofluorescence, and high UV autofluorescence. Consistent with these findings, we have found that the best results are obtained using Alexa Fluor® 555. Therefore, we have used this reagent for the first detection reaction using whichever probe gives the weaker signal. We were unable to resolve RNA signals in the UV channel because of high background autofluorescence.

Development of a Protocol for Multiplex FISH

In order to compare the TSA protocol to standard two-color *in situ* hybridization protocols, we performed FISH on 5–10 somite stage zebrafish embryos using the following markers for the developing hindbrain: *pax2a*-digoxigenin (DIG), *valentino* (*val*)-DIG, and *egr2b*-fluorescein. As shown in Figure 2, *pax2a* was detected at the mid-hindbrain barrier and otic placode, *val* was detected in rhombomeres 5 and 6, and *egr2b* was detected in rhombomeres 3 and 5, as has been shown previously.[7] Colocalization was most easily seen after flat-mounting embryos but both signals were also clearly visible in whole-mount embryos. A side-by-side comparison of FISH and WISH detection protocols show the increased capacity of FISH for colocalization of signals (Fig. 2A,B).

To demonstrate the specificity of this protocol as well as the capacity to colocalize signals within single cells, we performed double *in situ* hybridization using two signals that are known to be mutually exclusive, and two signals known to partially colocalize. In zebrafish, primitive macrophages appear just prior to circulation at 22 hours post fertilization (hpf) and express the *fms* gene encoding the macrophage colony stimulating factor receptor (M-CSF-R).[8] Early macrophages also express *l-plastin*, a gene-encoding leukocyte-specific plastin, a leukocyte actin-bundling protein expressed in macrophages and leukocytes during early development.[9] At 1 dpf, *fms* expression is also found in nonmelanogenic neural-crest–derived cells that do not express *l-plastin*. Therefore, the use of these two probes together should lead to a partial overlap of *fms* and *l-plastin* signals.[10] The myeloperoxidase gene *mpo* is also expressed by 1 dpf but is found only in neutrophils, and should be absent from macrophages.[11] Therefore, use of the *fms* and *mpo* probes should lead to completely nonoverlapping signals.

Double *in situ* hybridization was performed on whole 1-day postfertilization (dpf) embryos using DIG-labeled *fms* and fluorescein-labeled *mpo* RNA probes to determine whether or not two mutually exclusive signals could be separated using this protocol. As expected, both signals were found throughout the embryo, with the highest concentration found in the caudal vein area, which is the typical location of neutrophils and macrophages (data not shown). Higher magnification revealed that the *fms* and *mpo* signals can be completely separated (Fig. 3A). This experiment also demonstrates that signals from primaries derived from the same species can be completely separated by saturating the initial primary antibody with high concentrations of secondary antibody.

To determine whether signals can be colocalized, double *in situ* hybridization was performed on whole 1 dpf embryos using DIG-labeled *fms* and fluorescein-labeled *l-plastin* RNA probes. Individual cells could be identified with both signals, appearing yellow upon overlay (Fig. 3B). Expression of *l-plastin* is not limited to macrophages, and so individual *l-plastin*–positive and *fms*-negative cells were identified as expected. Thus, this procedure can be used to colocalize signals that appear within single cells.

To demonstrate that multiplex signals could be resolved within the embryo, we aimed to resolve a third color using TSA detection. TUNEL (terminal deoxynucleotidyl-transferase-mediated end labeling) reactions for labeling double-strand DNA breaks can be performed after *in situ* hybridization, making this an ideal procedure to test in conjunction with double FISH. TUNEL labeling is traditionally done as a marker for apoptosis and other forms of cell death that are known to occur at this stage.[12] As seen in Figure 4, all three signals can be readily resolved. As more haptens are tested and with the ability to label different antibodies using HRP-conjugated secondaries, it should be possible to image multiplex RNA and antibody signals using the wide variety of Alexa Fluor® TSA conjugates.

DISCUSSION

We have described a working procedure for two-color *in situ* hybridization on whole zebrafish embryos that allows for complete separation of nonoverlapping signals as well as colocalization of signals within single cells. In addition, this procedure can be combined with additional reactions such as the TUNEL reaction, to visualize multiple signals within single embryos. Using the specific reagents described in the experimental procedures, this procedure allows for identification of moderate and strong probes with minimal background staining.

The FISH protocol we have developed offers key benefits not available with color detection, such as colocalization of signals. It should be noted that these protocols were developed in older (3–7 dpf) embryos, which in our experience have more problems with probe penetration, background staining and autofluorescence than younger embryos. Therefore, both the sensitivity and specificity of the technique are likely to be better in younger zebrafish embryos that are studied by the majority of zebrafish researchers.

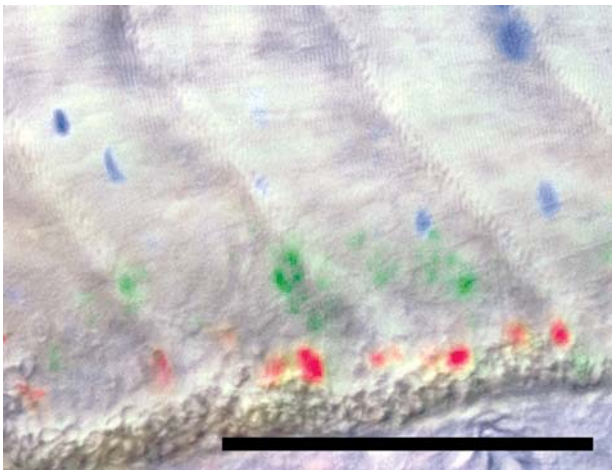

FIG. 4. Three color detection of two RNA signals and TUNEL-positive cells. Differential interference contrast (DIC) and fluorescent microscopy overlay of 5 days postfertilization (dpf) embryo tail (caudal vein at bottom) imaged for *fms*-DIG antisense RNA (red), *mpo*-fluorescein antisense RNA (green) and cells labeled with TUNEL enzyme (blue). Scale bar, 100 μm.

EXPERIMENTAL PROCEDURES

Bacterial cultures, fish embryo maintenance and infection were all performed as described previously.[13]

In Situ *Hybridization*

All steps were performed at room temperature unless otherwise specified. Embryos were fixed in 4% paraformaldehyde (PFA) for 12–16 hours at 4°C, dehydrated and stored in methanol (MeOH) for a minimum of 24 hours. Embryos were rehydrated in a graded series of MeOH/phosphate-buffered saline (PBS) washes and washed 4×5 minutes in PBS with 0.1% Tween 20 (PBT). Digestion was performed at 37°C in 10 μg/mL proteinase K for 5 minutes (5–10 somite embryos) or 30 minutes (1 dpf or older embryos), and embryos were postfixed in 4% PFA for 20 minutes and washed 5×5 minutes in PBT. Embryos were prehybridized in hybridization buffer (HB; 50% formamide, 5× SSC, 0.1% Tween 20, 50 μg/mL heparin, 500 μg/mL tRNA, and citric acid to pH 6.0) for 4–6 hours at 65°C. Probe was prepared by adding 100–200 ng of labeled RNA probe to 200 μL hybridization buffer. Embryos were hybridized with probe for 18 hours at 65°C; probe was reused 5–6 times without loss of signal. Probe was removed with a series of washes using SSC and PBT at 65°C: 1×15 minutes 66% HB/33% 2× SSC, 1×15

minutes 33% HB/66% 2× SSC, 1 × 15 minutes 2× SSC, and 2 × 30 minutes 0.2× SSC. After this, embryos were washed at room temperature: 1 × 10 minutes 66% 0.2× SSC/33% PBT, 1 × 10 minutes 33% 0.2× SSC/66% PBT, and 1 × 10 minutes PBT.

Antibody Detection of Labeled RNA Probes

Blocking buffer used was Roche Western block reagent (Roche 1921673, Roche, Nutley, NJ) diluted 1:10 in PBT, and all antibodies were diluted in blocking buffer. Embryos were washed for 2 hours in blocking buffer and incubated overnight with agitation at 4°C in 1:5000 dilution of primary antibody (sheep anti-DIG, Roche 1333089 or sheep antifluorescein, Roche 1426338). For our procedures, the anti-DIG antibody was applied first. After incubation with anti-DIG antibody, embryos were washed for 6 × 10 minutes in PBT, reblocked for 1 hour in blocking buffer, and incubated overnight at 4°C with agitation in 1:200 secondary antibody (HRP-conjugated rabbit anti-sheep, Jackson ImmunoResearch 313-035-047, Jackson ImmunoResearch, West Grove, PA). Embryos were washed 6 × 10 minutes in PBT and visualized using TSA. For detection of second signal after TSA reaction and inactivation, the procedure was repeated using the second primary antibody. Color detection was performed using BioRad AP Color Reagent (BioRad 170-6539, BioRad, Hercules, CA) and Sigma Fast® Fast Red tablet sets (Sigma F-4648, Sigma, St. Louis, MO), as specified by the manufacturers, and AP reactivity was stripped between colors using a 30 minute 0.1 M Glycine-HCl wash.

Tyramide Signal Amplification Detection

Tyramide reagent was used as specified by the manufacturer and diluted 1:100 in 1× amplification buffer (Molecular Probes T20912, T20917, T30952, and T30593). The reaction was performed for 15 minutes to 1 hour in the dark, and the embryos were washed 5 × 1 minute in PBT. The reaction was stopped with 1 × 30 minutes wash of 6% hydrogen peroxide in ddH_2O, which also helped eliminate any residual background signal.

Microscopy

DIC and video microscopy were performed using a Nikon E600 equipped with 10×, 20×, or 40× magnifications. Fluorescent images were collected with a Photometrics CoolSnap cf camera, and color images were collected with a Sony DKC-5000 digital camera Overlays of DIC and fluorescent images were produced by using Metamorph software as described.[13]

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