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Immunocytochemistry as a tool for zebrafish developmental neurobiology

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Abstract. Two methods are presented here that allow clear visualization of antibody localization in zebrafish whole mount preparations, both for immunocytochemistry (ICC) alone and in combination with *in situ* hybridization (ISH). The first protocol describes ICC performed using a modified permeabilization technique and the chromogen AEC (3-Amino-9-ethylcarbazole). The second protocol describes the co-localization of transcriptional and translational products using a combined ISH/ICC protocol. A fluorescing chromogen (Fast Red, FR) is used to detect mRNA transcripts by ISH, and is combined with ICC that uses a secondary antibody conjugated to a different fluorescent molecule (Alexa 488). These procedures allow the identification of gene expression patterns in cell types identifiable with known antibodies.

Key words: AEC (3-Amino-9-ethylcarbazole), Double *in situ* hybridization/immunocytochemistry, Fast Red, Immunocytochemistry, Zebrafish

1. Introduction

There are many factors that make the zebrafish an ideal developmental model to examine neurobiological processes [1]. This paper describes methods for the detection of proteins expressed in the vertebrate zebrafish nervous system during development.

The technique of immunocytochemistry (ICC) is not new to the zebrafish model. ICC protocols for zebrafish whole mounts have been extensively published, and many different chromogenic substrates have been used, including those relying on the PAP (peroxidase-antiperoxidase) and ABC (avidin-biotin complex) peroxidase techniques [for reviews see 2, 3]. Perhaps the most widely used substrate to detect the presence of a peroxidase molecule is DAB (diaminobenzidine), which gives a brown precipitate when in the presence of H_2O_2. However, DAB reactions often have significant background, which hinder localization of the specific antibody product. It is for this reason that we developed the use of another chromogenic substance for use with the peroxidase system in zebrafish whole mounts. We used 3-amino-9 ethylcarbazole (AEC) as our peroxidase substrate [4]. Furthermore, we optimized the penetration of antibodies in all stages of embryos using a permeabilization process that combines known techniques.

In addition, our experiments required the localization of both transcriptional and translational products. Thus, a double labeling protocol was developed that allowed fluorescent visualization of the mRNA and protein products. *In situ* hybridization (ISH) was performed using Fast Red (FR) as a substrate, which can be visualized by either bright field or epi-fluorescent microscopy. ISH was directly followed by an abbreviated ICC, and the antibody visualized by a fluorescing secondary antibody.

2. Materials

A. For ICC alone:
   - Collagenase – Sigma cat. C-9891.[1]
   - Normal Calf Serum – Gibco BRL cat. 26010-066.[2]
   - Primary antibody: anti-Hu (referred to here as HuA) – Molecular Probes cat. A-21271.[3]
   - Biotinylated horse anti-mouse secondary antibody and Avidin/Biotin complex found in the Vectastain ABC Elite kit – Vector cat. PK-6102.[4]
   - AEC (3-Amino-9-ethylcarbzole) – Sigma cat. A 5754.[1]
   - DMF (N,N dimethyl-formamide) – Sigma cat. D-4254.[1]

B. For ISH/ICC:
   - For ISH chemicals see [3].
   - Sigma *FAST* Fast Red TR/Napthol AS-MX tablets – Sigma cat. F 4648.[1]
   - Alexa 488 goat anti-mouse secondary antibody – Molecular Probes cat. A 11001.[3]

3. Procedures

ICC embryo preparation

Adult and embryonic zebrafish are raised according to the guidelines outlined in The Zebrafish Book [2]. All washes and incubations should be performed on a moving platform or nutator unless otherwise indicated. Permeabilization of the embryos is achieved by a tri-fold treatment. First, incubate embryos in distilled H_2O for 60 minutes at room temperature (RT, [2]). Next, place embryos in 100% acetone at –20 °C for 20 minutes [2]. Lastly, place embryos in collagenase (1 mg/ml) for a length of time appropriate for the embryo age [5, 6]. Younger embryos (19–24 hpf) require shorter incubation times (5–15 minutes) while older embryos and larvae (3–5 days post fertilization [dpf]) require longer incubation times (45–60 minutes). For collagenase treatment, embryos may be swirled in their vials instead of placing on a moving platform. Monitor the embryos closely at all times and remove the collagenase if it appears that embryos are beginning to fall apart. Stop collagenase digestion with several short washes in PBST, followed by 3 ten-minute PBST washes.

Primary antibody

Remove embryos from the glass vials and transfer to small plastic tubes (500 or 1,000 µl) to conserve solutions, which should cover embryos completely as well as fill most of the tube. Block embryos in 10% normal calf serum (NCS)/PBST for 1–4 hours and then incubate overnight at 4 °C in 10% NCS/PBST containing the primary antibody. The primary antibody HuA (mouse IgG2b isotype monoclonal anti-Hu) is used here at 1:1,000 from a stock of 0.9 mg/ml [7]. This antibody recognizes a neuron specific family of RNA binding proteins in vertebrates, involved in neuronal differentiation and survival of mature neurons [7]. HuA recognizes an epitope found on HuD, HuC and Hel N-1 Hu proteins in the rat [7]. In the zebrafish, HuA robustly labels Rohon-Beard (R-B) neurons at 24 hpf [6]. A transgenic GFP-HuC also shows expression of HuC in commissural interneurons in the spinal cord [8]. Related work using these techniques is published in Svoboda et al. [6].

Primary antibody detection

The next morning, wash embryos in PBST for at least 4 hours at room temperature (4 one-hour washes). Add the biotinylated, horse anti-mouse secondary antibody from the kit (1:250 dilution) and incubate overnight at 4 °C. The next day, wash embryos in PBST for at least 2 hours and incubate in avidin/biotin (A/B) solution for 40 minutes. After the A/B incubation, wash embryos in acetate buffer (50 mM, pH 5.0) for 2 hours (4 thirty-minute washes). Transfer embryos to multi-well staining dishes using a glass pipette. Mix a solution of fresh acetate buffer solution containing 0.04% AEC and 0.05% N,N dimethyl-formamide (DMF) and add 0.01% H_2O_2 to initiate the chromogenic reaction. Add the AEC solution to the embryos, monitor the reaction under a dissecting scope and have PBST readily available to stop the reaction. The AEC reaction occurs very quickly and forms a reddish-brown product. Within 2–10 minutes the AEC product should be evident and PBST added directly to the wells containing the AEC to stop the reaction. Remove the AEC/PBST in the wells and do 1–2 brief PBST washes to further stop the enzymatic reaction. Dispose of the AEC liquid in hazardous waste container. Continue to wash embryos in PBST for at least 60 minutes before visualization of the signal. Embryos can be stored in PBS or 50% glycerol/PBS at 4 °C.

Combined ISH and ICC

To visualize both transcriptional and translational products, a combined ISH/ICC protocol has been developed that utilizes fluorescent optics. To date, combined ISH/ICC protocols have only been described using chromogens detectable with light microscopy (for review see [3]).

Hybridization

Protocols for synthesizing nucleotide probes and performing zebrafish whole mount ISH have been published and will not be reviewed here (e.g. [3, 9]). The hybridization protocol utilized in these experiments was described by Schulte-Merker et al. [10]. However, our protocol utilizes Fast Red (FR) as the alkaline phosphatase substrate instead of nitroblue tetrazolium (NBT) with 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Detection of alkaline phosphatase signal

Detection of the alkaline phosphatase conjugated anti-digoxigenin Fab antibody (see [3]) is performed using the FR chromogen. Once the antibody has been removed with 4 thirty-minute washes in PBST, transfer embryos to 100 mM Tris, pH 8.4 for 3 five-minute washes on a moving platform. During the washes to remove the anti-digoxigenin probe, FR tablets need to be removed from the –20 °C freezer and allowed to warm to room temperature for at least 30 minutes prior to staining. Reconstitute the FR substrate tablets during the last Tris wash and add NaCl to a final concentration of 0.3 M to the staining solution to enhance the reaction [11]. Incubate the embryos in the FR solution for 2–48 hours at room temperature on the bench top and keep covered to avoid light exposure. The time required for devel-

opment of the signal will be probe specific. Once the signal has appeared, wash the embryos for 4 fifteen-minute rounds in PBST before beginning the ICC. If a red precipitate forms on the embryos, add a little more Tween-20 to your solution and continue washing. Embryos may be stored at 4 °C in PBST but best results occur when ICC is started immediately after the ISH protocol is finished.

ICC

Apply a blocking solution of 10% NCS/PBST to the ISH treated embryos for at least one hour at room temperature. The ISH procedure has already sufficiently permeabilized the embryos, so no further treatments are needed. All incubations and washes are done on a moving platform unless otherwise specified. Next, add the primary antibody, i.e. HuA, in 10% NCS/PBST overnight at 4 °C. The next day, wash at least 4 times with PBST for 30 minutes each. The secondary antibody is best applied in the afternoon. Add the secondary goat-anti-mouse antibody conjugated to Alexa 488 (1:500) for an overnight incubation at 4 °C. From this point forth, it is important to keep specimens covered to prevent bleaching of the fluorochromes. Wash specimens 4 times for 30 minutes with PBST the next morning and mount for microscopy.

Microscopy for ICC and ISH/ICC

Embryos can be visualized without perturbation of the embryo (i.e. as a whole mount) by placing on a slide in a pool of PBST. The embryos can also be squashed beneath a cover slip, but aqueous mounting media (glycerol or Gel/Mount) must be utilized as the AEC and FR products are alcohol soluble. In addition, the FR product and Alexa 488 (ICC label for the double) may be visualized using fluorescent optics with the appropriate filters.

All embryos shown here were viewed on a Nikon Eclipse TE2000 inverted scope using Bright Field or Epi-fluorescent (Rhodamine or Fluorescein) optics. Images were acquired digitally with a RT Spot Camera (Diagnostic Instruments).

4. Results and discussion

We modified existing protocols to develop a reliable and improved method for detection of cell markers in the developing zebrafish embryo nervous system. Our experience with ICC was similar to others and indicated that antibody penetration at later embryonic and larval stages is hindered due to the increased amount of connective tissue. Consequently, standard methods often provide inadequate permeabilization of whole mount preparations. To overcome this difficulty, we combined into a single protocol the various permeabilization strategies described in several existing methods. This allowed optimal permeabilization and access of antibodies to the preparation. Thus, our method is derived from existing protocols described by Gilmour [5] and those found in The Zebrafish Book [2].

ICC was performed on 24 hpf wild type zebrafish embryos using the HuA antibody. Similar results using the acetylated tubulin (aat) and zn-12 antibodies with older embryos (48–72 hpf) have been published in [6]. Zn-12 recognizes a carbohydrate epitope expressed on the cell surface, which is thought to be involved in axon guidance and cell recognition [12, 13]. Neuronal processes in both the CNS and PNS express zn-12, as well as R-B cells and many neurons in the hindbrain, including the Mauthner cell [2, 12, 13]. Aat recognizes the acetylated form of alpha tubulin [14]. In the zebrafish, aat has been shown to recognize many processes in the CNS [6, 15, 16].

Figure 1 reveals HuA staining in 24 hpf dorsal R-B neurons as revealed by the dark substrate produced by the AEC reaction. The dorsal view of the spinal cord reveals intensely stained R-B neurons most dorsally (arrows) and some less brightly labeled and deeper interneurons (arrowheads). Further, it reveals the specificity of the AEC substrate. Other spinal cord cells are not labeled non-specifically with the AEC. Similar results with 36 and 48 hpf embryos were obtained (data not shown), but 24 hpf embryos are shown, as HuA immunoreactivity is greater at earlier stages.

Thus, this novel combination of permeabilization and using AEC as a substrate has proven to be successful as an ICC tool in the zebrafish.

In addition to protein markers revealed by ICC, many mRNAs serve as useful markers of cell types in the developing zebrafish embryo. Thus, combining ICC with ISH allows for simultaneous study of 2 different molecular markers. ISH is always performed first, as mRNAs tend to be more labile than proteins in fixed embryo preparations [3]. The combined protocols utilize FR as the chromogenic substrate for ISH as it allows detection of transcriptional products at both the bright field and fluorescent levels [3]. However, instead of utilizing the avidin-biotin complex with AEC to visualize the antigen localization, a fluorescently labeled (Alexa 488) secondary antibody was used. In Figure 2, ISH was performed on zebrafish embryos with an antisense probe that recognizes a voltage-gated sodium channel (zNa$_v$1.6) expressed in trigeminal ganglia (TG) neurons [17]. We developed the double ISH/ICC to examine if a specific transcriptional product (i.e. zNa$_v$1.6 mRNA) was expressed ubiquitously within a specific population of cells (i.e. TG neurons expressing the HuA antigen). Detection of zNa$_v$1.6 mRNA was first examined in a lateral view of the TG with bright field optics (A). The same

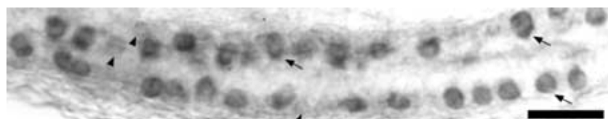

Figure 1. Dorsal spinal cord neurons in 24 hpf zebrafish embryos labeled with HuA. A dorsal view of the spinal cord reveals the AEC precipitate in R-B (arrows) and interneurons (arrowheads), while it is noticeably absent in other cells. Scale bar: 50 μM.

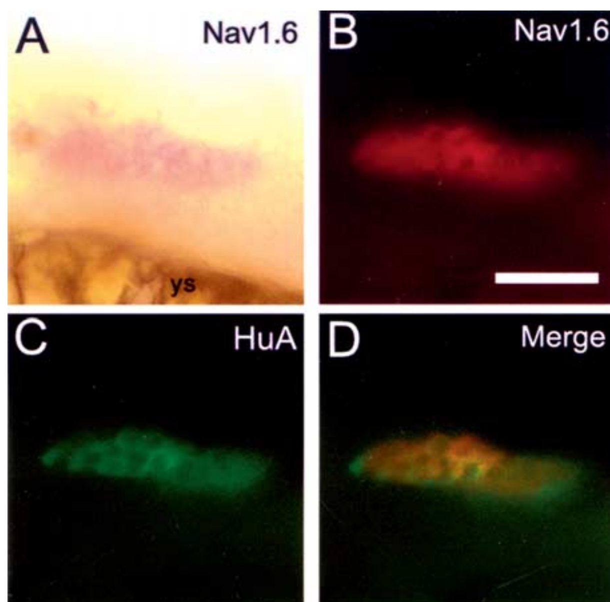

Figure 2. Trigeminal neurons in 24 hpf zebrafish embryos labeled with HuA and anti-sense probes to $zNa_v1.6$. Lateral views (ys: yolk sac) of the TG reveal bright field (A) and fluorescent (B) images of $zNa_v1.6$mRNA expression using FR as a substrate, HuA labeling with Alexa 488 (C), and merged images of HuA and $zNa_v1.6$ transcripts. These data demonstrate how the ISH/ICC protocol provides a way to examine the expression pattern of genes of interest (i.e. $zNa_v1.6$) within a population of cells known to express a specific cell marker (i.e. Hu). Scale bar: 50 μM.

transcriptional products can also be viewed using epi-fluorescence (rhodamine filter), which allows detection of some transcripts at levels too low to be seen in bright field (B). To allow further description of the neurons that express zNav1.6, localization of HuA was examined with epi-fluorescence (fluorescein filter, C).

Together, a merged image (D) of the translational and transcriptional products allowed identification (1) of the cells expressing $zNa_v1.6$ and (2) heterogeneity of these cells within the HuA population.

In summary, the two methods described here provide several advantages for study of neurobiological and developmental processes in zebrafish embryos and larvae. In our hands, these techniques provide consistent and reliable results with different antibodies. Moreover, using these methods, we obtained results with older embryos and larvae that were improved in comparison to results we obtained using published permeabilization techniques or alternate detection systems. Because the permeabilization techniques discussed here were developed to allow better penetration at older embryonic stages (48–120 hpf), they may not be necessary for younger stage embryos. Furthermore, we found that the ISH/ICC protocol provides a reliable way to look at two signals in the developing zebrafish with better results than other methods. For example, our technique provides less cross reactivity of the secondary antibodies than when double ICCs are performed with two monoclonal antibodies. Our ISH/ICC is also much easier to perform than double ISH, which can be technically challenging and has the potential for false positive signals when two alkaline phosphatase conjugated antibodies are used [18]. Antibodies to be used with the ISH/ICC protocol should be established by themselves before attempting the double procedure. Treatments with methanol, Proteinase K and multiple fixation steps of the ISH may disrupt the antigenic sites being recognized and thus the antibody may be incompatible for this procedure [18]. In some other cases, it may be possible that the FR precipitate occludes weaker antibody staining due to low protein levels, so care must be taken when choosing an antibody.

In the future, these techniques will continue to be useful as more zebrafish antibodies are developed which identify increasingly specific cell markers. As zebrafish genetics continue to allow identification of novel genes important for development in the nervous system, ICC and ISH/ICC will allow classification of the cells displaying these expression patterns. They will further aid in screening of fish for identification of mutant phenotypes and will support the identification of abnormalities that mimic those found in human diseases.

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Notes on suppliers

1. Sigma-Aldrich Corporation, St. Louis, MO, USA
2. Gibco BRL, 9800 Medical Center Dr., P.O. Box 6482, Rockville, MD, USA
3. Molecular Probes, 29851 Willow Creek Road, Eugene, OR 97402, USA
4. Vector Laboratories Inc., 30 Ingold Rd., Burlingame, CA 94010, USA

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