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Double labeling of neurons by retrograde axonal tracing and non-radioactive in situ hybridization in the CNS of adult zebrafish

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Abstract. A number of genes affecting axonal projections are currently being identified in zebrafish mutant screens. Analyzing the expression of these genes in the adult brain in relation to specific neuronal populations could yield insights into new functional contexts, such as the successful axonal regeneration in adult zebrafish. Here, we provide a relatively simple procedure for non-radioactive *in situ* hybridization in sections of adult zebrafish brains in combination with retrograde axonal tracing using the fluorescent neuronal tracer rhodamine dextran amine (RDA). A lesion is inflicted on the spinal cord of adult zebrafish and a crystal of RDA is then applied to the lesion site resulting in retrograde labeling of neurons in the brain through their spinal axons. Six to eighteen days later fish are perfusion-fixed, and *in situ* hybridization is carried out on vibratome-cut floating sections using a protocol simplified from that used for whole-mounted zebrafish embryos. This procedure leads to robust double labeling of axotomized neurons with RDA and an in situ hybridization signal for the growth-associated protein 43 (GAP-43). This method can be used to identify gene expression in specific populations of projection neurons and to detect changes in gene expression in axotomized neurons in the CNS of adult zebrafish.

Key words: Axotomy, *Danio rerio*, GAP-43, GFP, L1, NMLF, regeneration, transgenic

1. Introduction

The zebrafish is a powerful tool for elucidating gene function during development. Axonal projections, for example of retinal ganglion cells, have been the target of mutagenesis screens [1]. Genes from these mutant screens are being identified with increasing speed using positional cloning techniques. Analysis of the expression and regulation patterns of identified genes is often restricted to early development, even though analysis of these genes in late larval or adult brains could yield important insights into the same or different functional contexts from those apparent in early development. However, the anatomy of the adult brain is more complex than that of embryos or early larvae [16]. Producing specific antibodies that could reveal axonal projection patterns, as well as aid in the identification of cells expressing a gene of interest, is time consuming and success is not guaranteed. In situ hybridization of the adult brain can show whether a specific mRNA is expressed. However, it may be difficult to identify the cell type that expresses a gene of interest by the relative position of the labeled somata alone. Combining in situ hybridization with neuroanatomical tracing can help to characterize neuronal populations that express a certain gene by their axonal projection patterns. A robust protocol for simultaneous neuroanatomical tracing and in situ hybridization can thus help to extend mutant analysis to juvenile and adult zebrafish.

Another aspect that makes adult zebrafish an interesting model system is their ability to regenerate severed axonal connections in the CNS, in contrast to mammals [for review, see 4]. In addition, access to regeneration-related genes is relatively easy in zebrafish, due to the genome sequencing project in this species. Moreover, cDNA microarrays are becoming available for zebrafish [10]. These could be used to determine expression profiles of neurons with regenerating axons vs. non-regenerating neurons. Mutant and transgenic zebrafish are being produced and can be analyzed in terms of gene activation during development and regeneration [7, 13]. Thus, the adult zebrafish is a model system for successful adult CNS regeneration that is of considerable interest in the near future.

For regeneration studies, it would be very interesting to determine which neurons have been axotomized and what the expression status of certain genes is in the same neurons after a lesion. For example, a lesion to the spinal cord may lead to axon regrowth and activation of regeneration-related genes in populations of neurons that have their cell somata remote from the lesion site, such as neurons that have their somata in the brain and their axons in the spinal cord [2]. Such neurons can be identified by applying a neuronal tracer to the lesion site. The tracer is

selectively transported in severed axons and is retrogradely transported to the cell somata located in the brain. Thus, the tracer defines the population of neurons with axons that have been severed by a lesion [3]. Performing *in situ* hybridization for genes of interest in the same preparations may then show whether projection neurons in brain nuclei react to a lesion by dysregulating these genes. Gene regulation can thus be directly compared between axotomized and non-axotomized neurons, for example after a hemisection of the spinal cord, in the same brain nuclei of the same animals. Here we describe a relatively simple and robust method for combining retrograde axonal tracing with *in situ* hybridization in adult zebrafish brains.

2. Materials

Laboratory chemicals were purchased from Sigma (Deisenhofen, Germany).

Rhodamine dextran amine (RDA; MW 10,000) was purchased from Molecular Probes (Leiden, Netherlands).

The Megascript™ system for digoxygenin (DIG)-labeling of cRNA probes was purchased from Ambion (Huntington, Great Britain).

The anti-DIG Fab fragment antibody and the Roche blocking reagent were purchased from Roche Diagnostics (Mannheim, Germany).

Cell culture dishes (24 wells) were purchased from Techno Plastic Products (Trasadingen, Switzerland).

3. Procedures

Animals

Adult (body length > 2 cm, age > 4 months) zebrafish, *Danio rerio*, were bought from a local fish breeder. Prior to surgery, adult fish were kept in groups of 10 animals at a 14 hour light and 10 hour dark cycle and a temperature of 27 °C. After surgery, individual fish were kept in two liter tanks. Fish were fed dried fish food and live brine shrimp. All animal experiments were approved by the University and State of Hamburg animal care committees and conformed to NIH guidelines.

Spinal cord transection and tracer application

Spinal cord transection was performed as previously described [3]. Briefly, fish were anesthetized by immersion in 0.033% aminobenzoic acid ethyl-methylester (MS222, Sigma) for 5 minutes. A longitudinal incision was made at the side of the fish to expose the vertebral column. The spinal cord was cut completely between two vertebrae at the level of the opercula, corresponding to the brainstem/spinal cord transition zone. A small piece of re-crystalized RDA, approximately half the size of the spinal cord diameter, was immediately placed into the lesion site to avoid resealing of severed axonal membranes before axons were able to take up the tracer. After surgery, wounds were closed with histoacryl (B. Braun Melsungen, Germany) and the fish were kept for 6 to 18 days before they were sacrificed by an overdose of MS222 (0.1%). This time interval allows for retrograde transport of the tracer and for upregulation of growth-related genes to occur in lesioned neurons. Fish were transcardially perfused with 4% paraformaldehyde. Brains were dissected free and post-fixed in 4% paraformaldehyde overnight before they were processed for *in situ* hybridization. To control whether neurons were specifically traced through their severed spinal axons at the tracer application site, RDA was inserted into the trunk muscle tissue at the level of the brainstem/spinal cord transition zone next to the spinal cord with the spinal cord left intact. A horizontal incision into the skin and musculature was made at the level of the spinal cord, and the muscle tissue was pushed aside with fine forceps to insert the RDA crystal. The tracer was left in the fish for 6 days. This procedure did not label any cells in the brain.

Digoxygenin-labeling of cRNA probes

Digoxigenin (DIG) – labeled RNA sense and anti-sense probes were generated using the Megascript™ system (Ambion) according to the manufacturer's instructions. The template for the GAP-43 probe has been described elsewhere [11]. Anti-sense and sense probes were transcribed from the same plasmid using the appropriate primers yielding probes of 1 kb in length. One µg of plasmid DNA yielded 20 µl of labeled probe. Optimal probe dilutions (in hybridization buffer, see below) were directly titrated in *in situ* hybridization experiments and were between 1:800 to 1:4,000. Immediately before use, the diluted probe was heated to 80 °C and rapidly cooled on ice. It was found that alkaline hydrolysis was not necessary for tissue penetration. We successfully used this method for probes of up to 4 kb in length. Although not systematically tested, it was our impression that signals developed more quickly and strongly with longer probes. Thus, whenever possible, we used probes covering the entire cDNA of a gene of interest.

Combined tracing and in situ hybridization

To combine axonal tracing with *in situ* hybridization in the same tissue sections, free-floating sections of RDA labeled brains were used for *in situ* hybridization. Fixed brains of animals that received RDA at a spinal cord transection site (see above) were embedded in 4% agar and cross sectioned (50 µm

in thickness) on a vibratome (Leica, Bensheim, Germany). Sections were collected in small containers with a meshwork bottom filled with phosphate buffered saline (pH 7.4; PBS). These containers are built from cross sections of 10 ml plastic syringes with a mesh (0.115 mm × 0.08 mm mesh width, stainless steel, Dietel, Hamburg, Germany) molten onto one opening (on a hot plate, 200 °C), through which sections cannot be lost. Containers are small enough to be placed into 24 well cell culture dishes. To ensure that neurons in the brain have been retrogradely labeled, some sections can be removed and mounted directly in glycerol at this stage. RDA labeling can be observed under a fluorescence microscope.

For subsequent *in situ* hybridization, we used a protocol that is a simplified version of that used for whole-mount *in situ* hybridization of zebrafish embryos [15] to process free-floating sections. Each change of solutions was carried out by transferring the small containers from one well to the next using a pair of forceps. All steps were carried out at room temperature unless indicated otherwise. Sections were washed in PBS/0.1% tween-20 (PBST) for 5 minutes and incubated with proteinase K (10 µg/ml in PBST) for 9 minutes, rinsed briefly twice in glycine (2 mg/ml) in PBST, post-fixed in 4% paraformaldehyde in PBS for 20 minutes and washed 4 times in PBST for 5 minutes each.

For prehybridization, sections were washed in hybridization buffer [5 ml formamide; 2.5 ml 20 × SSC; 10 µl Tween-20; 100 µl of a yeast RNA stock solution (100 mg/ml); 2.4 ml H_2O; 10 µl heparin stock solution (50 mg/ml); all components made up RNAse-free with diethylpyrocarbonate-treated water] once for 5 minutes and were then incubated in hybridization buffer for 3 hours at 55 °C. Hybridization was carried out in hybridization buffer containing the DIG-labeled probe at 55 °C overnight. Sections were washed twice in a buffer comprising half formamide and half 2 × SSC/0.1% Tween-20 (SSCT) at 55 °C for 30 minutes each time, once in 2 × SSCT at 55 °C for 15 minutes and twice in 0.2 × SSCT at 55 °C for 30 minutes each. Sections were blocked in Roche Blocking Reagent at room temperature for one hour. Detection was carried out at 4 °C overnight with an alkaline phosphatase-coupled anti-DIG Fab fragment antibody (Roche), diluted 1:2,000–1:8,000 in Roche Blocking reagent (Roche). After 6 washes in PBST for 20 minutes each, the signal was developed in staining solution [one nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate tablet (Sigma, B-5655) in 10 ml H_2O, yielding a final concentration of 0.15 mg/ml BCIP, 0.3 mg/ml NBT, 0.1 M Tris, 5 mM $MgCl_2$] for 10 minutes to overnight. Signal development was observed under a stereomicroscope. When a reaction product was clearly visible, signal development was stopped by several washes in PBS. Sections were removed individually from their containers using small paintbrushes and mounted in 90% glycerol (technical grade) containing 10% PBS and 2.5% sodium iodide to stabilize fluorescence. Tissue sections from unlesioned and lesioned animals were processed in parallel using the sense and anti-sense GAP-43 cRNA probes described above. Using fluorescence and transmitted light, co-localization of tracer and *in situ* hybridization signal in individual tissue sections was evaluated. As a control for the specificity of the *in situ* hybridization signal, sections were incubated with GAP-43 sense cRNA probes, which did not yield any signal (Figure 1E). Results were documented using a Zeiss (Oberkochen, Germany) Axiophot with attached digital camera equipment (Axiocam, Zeiss) using 10× and 100× objectives. Photographic plates were prepared using Adobe Photoshop.

4. Results and discussions

After tracing, in combination with *in situ* hybridization, both the *in situ* hybridization and tracer signals were clearly visible (Figure 1A, B). RDA reliably labeled neurons in all of the identified twenty brain nuclei with spinal projections in zebrafish [3]. The retrograde tracer was found primarily in the somata of neurons (Figure 1D, F). Fluorescence was diffuse with interspersed bright granules, which is characteristic of RDA labeling. Proximal dendrites were also labeled (Figure 1D). Intense GAP-43 *in situ* hybridization signal was also found in these brain nuclei, overlapping with the retrograde tracer signal in the cytoplasm of the neurons as a purple reaction product (Figure 1C, D). Labeling patterns from either RDA tracing or GAP-43 *in situ* hybridization alone were not different from those observed in double labeling procedures and confirmed those previously reported [2, 3]. The fact that both the tracer signal and the *in situ* hybridization signal were highly localized to the specific brain nuclei that were expected to be labeled indicates that the double-labeling procedure did not produce additional artefactual signals. In control double labeling experiments, using a GAP-43 sense RNA probe, the fluorescent tracer signal, but no *in situ* hybridization signal, was detected (Figure 1E, F). In brain nuclei projecting to the spinal cord of unlesioned animals no fluorescence signal, and only very few GAP-43 mRNA positive cell profiles, were detected. The low expression level of GAP-43 mRNA in unlesioned animals indicates that expression of GAP-43 was increased after a spinal lesion [2]. Using probes to cell recognition molecules such as L1.1 [2], produced similar results to those shown here for GAP-43 (not shown). The *in situ* hybridization signal usually developed within 10 minutes, compared to weeks needed for radioactive *in situ* hybridization [6]. Given that the

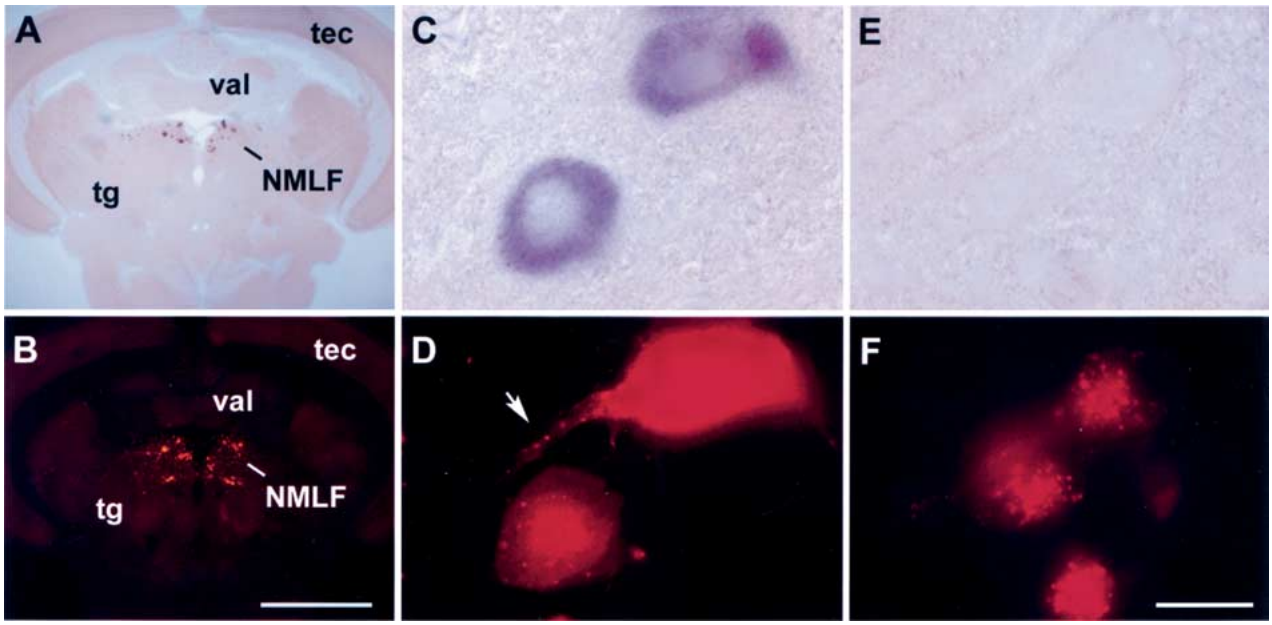

Figure 1. *In situ* hybridization for GAP-43 mRNA combined with retrograde tracing of neuronal somata in the same tissue sections. **A, B**: A cross section at low magnification through the midbrain of an adult zebrafish, cells labeled for GAP-43 mRNA are observed in the nucleus of the medial longitudinal fascicle (NMLF) 6 days after spinal cord transection (A). Cells in the NMLF are retrogradely labeled by RDA in the same tissue section (B). tec = optic tectum; val = valvula cerebelli; tg = tegmentum. **C, D**: A cross section through the brainstem of an adult zebrafish is shown at high magnification; dorsal is up; lateral is left. In the same tissue section, two cells in the superior reticular formation are double labeled for GAP-43 mRNA (C) and RDA (D). RDA also labels the proximal dendrite of one cell (arrow in D), while the *in situ* hybridization signal is mostly restricted to the soma. **E, F**: Hybridization with a sense probe to GAP-43 yields no signal (E) in cells of the superior reticular formation that are retrogradely labeled by RDA (F) in the same tissue section (Orientation as in C, D). Bar in B = 750 μm for A,B; bar in F = 25 μm for C-F.

alkaline phosphatase used in our protocol is active for several hours and enzymatic reaction kinetics are linear for some time, we assume that potentially less abundant mRNAs can be detected by simply prolonging the developing time of the non-radioactive *in situ* hybridization. Both the *in situ* hybridization signal and the fluorescent tracer signal were stable for at least a year in our hands. The fluorescence signal can be photographed several times under a conventional epifluorescence microscope at magnifications of 10–100 fold without major loss of intensity through bleaching.

Combining *in situ* hybridization with a dark precipitate signal and tracing with a fluorescence signal in the cytoplasm entails the danger of the fluorescence signal being quenched by the dark precipitate. However, comparing the RDA signal before the *in situ* hybridization with that after the *in situ* hybridization showed virtually no reduction in the fluorescence signal, even though the *in situ* hybridization signal was strong (Figure 1A, C). The retrograde tracer labels proximal dendrites in addition to somata, whereas the *in situ* hybridization signal is mostly restricted to the somata. If quenching effects were strong, one would probably have observed quenched fluorescence in the somata of neurons with fluorescence still present in proximal dendrites, but this was not the case. Counting of cell profiles labeled by RDA in select brain nuclei did not show any systematic differences between animals that only received RDA and those in which *in situ* hybridization was additionally performed (data not shown), again indicating that quenching was not a matter of concern. Nevertheless, when testing a new *in situ* hybridization probe, it should be examined whether or not fluorescence signal occurs in proximal dendrites, but not in neuronal somata. Alternatively, cell profile counts can be compared between single and double labeling procedures. If quenching occurs, probe concentration or developing time can be reduced in order to reduce the amount of precipitate formed.

It is unlikely that the presence of the tracer in the cytoplasm negatively affects the *in situ* hybridization, since the GAP-43 mRNA signal was found primarily in the cells that had been retrogradely traced and was also detected in cells showing a very bright fluorescence signal. In fact, in specific brain nuclei, up to 90% of the retrogradely labeled neurons were also labeled by the *in situ* hybridization signal (Figure 1; own unpublished observations). This was expected since it has been shown that axotomized neurons in these brain nuclei undergo axon regrowth, which goes along with increased expression of growth-related molecules [3]. The double labeling procedure developed here can directly show which genes indi-

vidual neurons express following axotomy, in this case GAP-43.

We chose RDA as a tracer because it is robust enough to withstand the relatively harsh treatment used for *in situ* hybridization. Moreover, this fluorescent dextran can be directly observed without the need for histochemical signal development. RDA accumulates in the neuronal somata, which makes it a sensitive retrograde tracer. The tracer is not only useful for labeling neurons with spinal axons: we could show that by applying RDA to the trunk musculature, spinal motor neurons and neurons in dorsal root ganglia are also labeled (own unpublished observations). The tracer could also be applied locally to different parts of the brain to trace connections within the brain. A limitation to the use of RDA is that locations deep within the brain may not be easily accessible in the living animal. Lipophilic tracers, such as DiI, can be used on fixed brain tissue of teleost fish [9, 12]. Here we use RDA as a retrograde tracer, i.e. axons are filled from a lesion site and the tracer is transported from the severed axon to the soma and the proximal dendrites. While RDA labels somata very reliably, it does not always label axons and axon terminals in anterograde tracing in great detail. Lower molecular weight dextrans (3,000 MW dextrans are available) are reportedly transported more rapidly and may label axons in greater detail [5]. These could be more suitable for anterograde tracing. Another tracer that can be used for anterograde tracing is biocytin, which labels axonal morphologies, including terminals, in Golgi-like detail [3]. Even though the fluorescence of RDA is very stable, the signal is not visible in normal light microscopy. To obtain a tracer signal that is visible in light microscopy, biotinylated dextrans, which should have similar tracing properties as RDA, are available. Horseradish peroxidase is another simple and reliable retrograde tracer for light microscopy in the CNS of adult zebrafish [3, 14].

The double labeling method described here could also be used in transgenic fish, which express green fluorescent protein (GFP) in specific neuronal populations. Transgenic zebrafish lines have been produced, in which the activation of regeneration-related genes after a lesion of the adult CNS is analyzed by the expression of GFP under the control of gene-specific promoters [7, 13]. These fish, in conjunction with *in situ* hybridization/tracing, could be used to simultaneously analyze the regulation of more than one gene in axotomized neurons. Neuronal tracing has been used to distinguish subpopulations of GFP-positive motor neurons by their projection patterns in developing zebrafish [8]. Using the double labeling method presented here, these subpopulations could be further characterized by showing possible differences in gene expression that correlate with differences in axonal projection patterns.

In conclusion, we present a rapid, reliable double labeling method for identifying neurons by their axonal projection pattern and their expression status of genes of interest in adult zebrafish brains. RDA as a neuroanatomical tracer is stable for at least one year and can be detected without secondary detection procedures using fluorescence microscopy. The signal from non-radioactive *in situ* hybridization is likewise stable and rapidly developed. Therefore, we believe that this method will be useful to researchers wishing to analyze neuronal gene expression and connectivities in the CNS of adult zebrafish.

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