Spatio-temporal gene expression analysis from 3D in situ hybridization images

Welten, M.C.M.

Chapter 2

ZebraFISH: Fluorescent *in situ* hybridization protocol and 3 D imaging of gene expression patterns.

M.C.M. Welten [1,2], S.B De Haan[1,2], N. Van den Boogert [1], J.N. Noordermeer [3], G.E.M. Lamers [2], H.P. Spaink [2], A.H. Meijer [2] and F.J. Verbeek.[1]

1. Imagery and Media, Leiden Institute of Advanced Computer Science, Leiden University, Niels Bohrweg 1, 2333 CA Leiden, the Netherlands
2.Institute of Biology, Leiden University, Clusius Laboratory, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands
3.Leiden University Medical Center, Developmental neurobiology group, Dept. of molecular cell biology, Wassenaarseweg 72, 2300 RA Leiden, The Netherlands

Originally published in Zebrafish, 2006: Vol. 3, No. 4: 465-476
Modified and updated version

.

A Tool to visualize gene expression

ABSTRACT

We present a method and protocol for fluorescent *in situ* hybridization (FISH) in zebrafish embryos to enable three-dimensional imaging of patterns of gene expression using confocal laser scanning microscopy. We describe the development of our protocol and the processing workflow of the three-dimensional images from the confocal microscope. We refer to this protocol as zebraFISH. FISH is based on the use of tyramide signal amplification (TSA), which results in highly sensitive and very localized fluorescent staining. The zebraFISH protocol was extensively tested and here we present a panel of five probes for genes expressed in different tissues or single cells. FISH in combination with confocal laser scanning microscopy provides an excellent tool to generate three-dimensional images of patterns of gene expression. We propose that such three-dimensional images are suitable for building a repository of gene expression patterns, complementary to our previously published three-dimensional anatomical atlas of zebrafish development (bio-imaging.liacs.nl/). Our methodology for image processing of three-dimensional confocal images allows an analytical approach to the definition of gene expression domains based on the three-dimensional anatomical atlas.

INTRODUCTION

The zebrafish is an excellent model system for developmental and molecular genetics, for functional analysis of genes, as well as to gain understanding of genetic networks involved in human disease (Stern and Zon, 2003).The basis for such analysis is the *in situ* study of gene expression. The best tool for studying spatial characteristics of patterns of gene expression is *in situ* hybridization (ISH) (Wilkinson, 1998). In zebrafish research it is, in most cases, applied on whole-mount embryos, using digoxigenin-labeled antisense RNA probes and the alkaline phosphatase (AP) detection method (Wilkinson, 1998). A high resolution ISH protocol with the AP detection method for high throughput genomic screens that utilize spatial patterns of gene expression as readout has been developed by Thisse et al. (Thisse et al, 1993, 2004). Patterns of gene expression are then compared by microscope images of the whole-mount ISH, providing two-dimensional information; excellent results and observations have been shown (Thisse et al, 1993). For analytical approaches, a true three-dimensional spatial representation of the pattern of gene expression is required (Verbeek et al, 1999). Three-dimensional images can be acquired by using the confocal laser scanning microscope (CLSM). Up to a certain age, zebrafish embryos and larvae can be very well visualized with CLSM and true three-dimensional images of high resolution can be produced. With CLSM, visualizing a pattern of gene expression is based on tagging a fluorescent molecule such as Cyanine 3 or Cyanine 5 (Cy3 or Cy5, PerkinElmer) to the RNA probe.

We argue that a suitable protocol for fluorescent *in situ* hybridization (FISH) can easily be adapted from existing protocols and be used in high-throughput applications in zebrafish (zebraFISH). To adapt this protocol as much as possible to common laboratory practice of the zebrafish researcher as well as to high-throughput screening, we used the standard ISH AP detection protocol developed by Thisse et al. (Thisse et al, 1993, 2004) as a foundation. Whole-mount FISH has been successfully applied in *Drosophila* (Kearny et al, 2004; Paddock, 1999) and the protocol used in the *Drosophila* community was taken as the starting point to develop and optimize the fluorescent labelling steps for zebraFISH. The first steps of our FISH protocol are identical to the standard Thisse (Thisse et al, 1993, 2004) protocol and it diverges at the point of application of the fluorescent substrate. The protocol uses an amplification step in order to obtain sufficiently high signal-to-noise ratios for visualization and localization for three-dimensional imaging. This amplification step, achieved by the tyramid signal amplification (TSA) technique, is an essential ingredient in our zebraFISH protocol; the result is an amplification of the Cy3/Cy5 signal. Horseradish peroxidase (HRP) is used to catalyze the deposition of a fluorophore-labelled tyramide amplification reagent at the site of probe binding. This approach is, in a way, complementary to AP detection substrates (i.e., NBT/BCIP) that precipitate diffusely near the location of gene expression. In TSA, a relatively short defined staining procedure provides as strong expression signal with relatively low background staining (Zaidi et al, 2000). Even weak probes and small gene expression domains can be visualized using our approach for TSA detection. Independent of our research and testing of the zebraFISH protocol, another protocol for fluorescent *in situ* hybridization, also based on TSA, has been developed by Clay and Ramakrishnan (2005). The focus of their protocol was on multiplex detection of genes with overlapping expression patterns, with AlexaFluor conjugates used to

demonstrate colocalization expression within single cells. In the zebraFISH protocol described here, we also use multiple fluorescent labels. The purpose is however different. In order to reveal both outline and anatomical structures of the embryo, we combine TSA/Cy3 or TSA/Cy5 staining with a nuclear counter staining, using SYTOX Green or SYTOX Orange. In this way the gross texture of the embryo is visualized by clusters of nuclei. The interpretation of the three-dimensional image is greatly facilitated in this manner and we use this to colocate anatomical domains with respect to the expression patterns. Our protocol differs from that of Clay and Ramakrishnan in that we use a one-step antibody detection procedure (HRP-conjugated anti-dioxigenin antibody) instead of a two-step detection procedure that uses sheep anti-dioxigenin and HRP-conjugated anti-sheep antibodies.
Our one-step approach results in a procedure that is one day shorter; this is a major advantage for high-throughput applications. In this paper we demonstrate that our zebraFISH protocol with one-step TSA detection provides adequate sensitivity to detect expression of a variety of developmental marker genes, such as *myoD*, *krox20*, *otx2*, *pax2.1*, and *mpx.*

MATERIALS AND METHODS

Zebrafish maintenance and embryonic staging

Embryos were collected from a laboratory breeding colony of albino zebrafish kept at 28°C on a 14:10 h light/dark rhythm and raised under standard conditions (zfin.org). Embryos were staged at 28°C according to hpf and morphological criteria (Kimmel et al, 1995).

cDNA clones and RNA probe synthesis

cDNA clones of *myoD*, *krox20*, *otx2*, and *pax2.1* were provided by J. Bakkers (NIOB, Utrecht, the Netherlands). An *mpx* cDNA clone (BC056287) was obtained from RZPD (Berlin, Germany). Antisense riboprobes labeled with digoxigenin-11-UTP (Roche) were synthesized from linearized cDNA clones using T7, T3, or Sp6 RNA polymerases (Maxiscript kit, Ambion) according to the manufacturer's instructions.

Fluorescent in situ *hybridization (FISH)*

In brief, embryos were manually dechorionated, fixed overnight in 4% buffered paraformaldehyde (PFA) at 4°C, dehydrated through a graded methanol series and stored at -20°C in methanol. Endogenous peroxidase activity was inhibited by incubation in 3% H_2O_2 in methanol for 20 min at room temperature. Embryos were rehydrated through a graded methanol series to 100% PBST (phosphate buffered saline, pH 7.0, containing 0.1% Tween 20) and permeabilized with 10 ug/ml proteinase K (Promega) in PBST at 37°C from 15 min (24 hpf embryos) to 30 min (36–120 hpf embryos). To stop the reaction, the embryos were washed in PBST for 5 min. Embryos were refixed in 4% buffered PFA and washed 5 times in PBST for 5 min. Next, the embryos were prehybridized for 2–5 h at 55°C in hybridization buffer containing 50% formamide, 5xSSC(20xSSC = 3M NaCl, 300 mM trisodium citrate), 0.1% Tween 20, 500 ug/ml tRNA (Sigma), and 50 ug/ml heparin (Sigma), pH 6.0–6.5. Hybridization was carried out overnight at 55°C in 200 ul hybridization buffer containing 50–100 ng of digoxigenin-

labelled riboprobe. After hybridization, the embryos were briefly washed in hybridization buffer (without tRNA and heparin) and next washed at 55°C in 15-min steps over a gradient of hybridization buffer and 2xSSC (75%/25%, 50%/50%, 25%/75%) to a final wash in 100% 2xSSC. Subsequently, the embryos were washed 2 times in 0.2x SSC for 30 min at room temperature and washed in 10-min steps at room temperature over a gradient of 0.2xSSC and PBST (75%/25%, 50%/50%, 25%/75%) to a final wash in 100% PBST. Embryos were preabsorbed for 2 h at room temperature under slow agitation in antibody buffer consisting of PBST containing 2% sheep serum and 2 mg/ml bovine serum albumin. Meanwhile, anti-DIG-HRP antibody (anti-DIG-POD, Roche) was diluted 1:1000 in antibody buffer and preadsorbed for 2 h at room temperature under gentle agitation. After pre-incubation, the antibody buffer was replaced by the preadsorbed 1:1000 diluted anti- DIG-HRP solution and embryos were incubated overnight at 4°C under gentle agitation. After antibody incubation, embryos were washed 6 times for 15 min in PBST and stained with TSA/Cy3 reagent (PerkinElmer) diluted 1:50 in amplification buffer (provided with the TSA/Cy3 amplification kit) for 30 min at room temperature. After staining, embryos were washed 8 times for 15 min in PBST, and a nuclear counterstaining with 100 nM SYTOX Green (Molecular Probes) was performed for 30 min. Embryos were washed 6 times for 15 min in PBST. For microscopy, embryos were mounted and stored in Gelvatol containing 100 mg/ml DABCO (1,4-diazabicyclo[2.2.2]octane) in a glass-bottom dish. (See Appendix for complete protocol.) For control of the *in situ* reaction and comparison with FISH results, probe detection with alkaline-phosphatase-conjugated anti-digoxigenin (Roche) and nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate substrate (NBT/BCIP, Roche) as described by Thisse et al.(1993, 2004) was carried out in parallel. Each control ISH was examined on a stereo microscope and photographed with a digital camera for later reference.

Microscopy and image processing

Images of NBT/BCIP-stained embryos were acquired using a Leica MZFL-III12 stereomicroscope equipped with a Leica DC 500 digital camera. Confocal imaging of embryos was performed using a Leica TCS/SP DM IRBE confocal laser scanning microscope (inverted setup) equipped with an Ar/Kr laser. Excitation and emission wavelengths of used fluorophores are summarized in Table 1.

Table 1. Overview of fluorophores, excitation, and emission spectra described in ZebraFISH.

Fluorophore	*Excitation (nm)*	*Excitation in zebraFISH protocol (nm)*	*Emission (nm)*	*Emission in zebraFISH protocol (nm)*
Cyanine 3	550	568 (Kr laser)	570	580
Cyanine 5	648	NA	667	NA
ELF	345	350 (Zeiss Axioplan)	530	NA
SYTOX Green	504	488 (Ar laser)	523	523
SYTOX Orange	547	NA	570	NA

In our setup, excitation resulted in a green and a red channel. All images shown were obtained with a 10x plan apo lens with a large working distance (NA 0.24). The images are sampled isometrically, taking the resolution in the xy plane as the guide for the z-axis sampling; each image slice is sampled to 1024 x 1024 pixels. The CLSM images are saved as two-channel multiple TIFF files and these files were processed with dedicated image processing software (Bei et al.2006).

The three-dimensional reconstructions (see Fig. 1) were produced from the CLSM images using the TDR-3Dbase annotation and reconstruction software (Verbeek et al. 2000; Verbeek, 2000). By means of the TDR- 3Dbase software, gene expression and anatomical domains in the three-dimensional images were traced, either manually or via automated procedures, to result in the three-dimensional models.

RESULTS

To test and optimize the zebraFISH protocol, we used a panel of 5 probes which we consider represent a range of patterns, from expression in single cells (*mpx*) to expression in different tissues of the brain (*krox20*, *otx2*, *pax2.1*) and in the somites (*myoD*). Figures 1 and 2 present an overview of the results with the panel of 5 genes. In Figure 1, results of both chromogene and TSA detection are shown for each of these genes. In all cases, the TSA/Cy3-based detection FISH expression patterns perfectly corresponded to those obtained through standard AP detection. In agreement with previous reports, at 24 hpf, *MyoD* expression is detected in the somites (Weinberg et al, 1996), *krox20* is expressed in rhombomeres 3 and 5 (Woo and Fraser, 1998), *otx2* is expressed in the diencephalon and mesencephalon (Mercier et al, 1995), and *pax2.1* shows expression domains in the midbrain-hindbrain boundary, the optic stalk, and the otic vesicle (Lun and Brand, 1998). In addition to the three-dimensional images, we provide three-dimensional reconstructions for these four expression patterns, using the three-dimensional image stack as input to our three-dimensional reconstruction software, TDR-3Dbase (Verbeek et al, 2000; Verbeek, 2000).

The reconstruction process results in a geometric model through which the expression patterns and some of the surrounding tissues can be visualized (Fig. 1D). These three-dimensional visualizations give further insight into spatial relationships of the pattern and the three-dimensional models underlying the visualizations can be used for quantitative analysis. TSA/Cy 3 detection reveals clear and specific expression patterns even for genes expressed in small domains, as illustrated in particular by the *mpx* expression pattern (Fig. 2). Lieschke et al (2001) reported previously that neutrophil granulocytes

expressing mpx are scattered over the yolk sac, the head region, and the ventral venous plexus of the 48 hpf embryo. TSA/Cy3 detection clearly revealed the expression pattern in single cells.
In order to obtain optimal and reproducible results from zebraFISH we examined several important parameters of the procedure. From laboratory practice we have learned that some fine tuning is required for the critical steps in every probe.

Embryo culture
The addition of methylene blue during embryo culture is often used to prevent fungal growth; however, it also induces autofluorescence. Therefore, embryos should be grown in egg medium (www.zfin.org) without methylene blue.

In situ hybridization
One of the crucial parameters of the zebraFISH protocol is to adjust the pH of the prehybridization and hybridization mix to 6–6.5. All our hybridizations were carried out at 55°C. In our experiments, hybridization at this temperature yielded the best signal and the most stable signal complex; for standard ISH, Thisse et al. (1993, 2004) suggest hybridization at 70°C.

Antibody incubation and blocking reaction
If the background is too high, lower the antibody concentration. Titration of the used antibody is recommended by the manufacturer (Roche) to obtain the optimal antibody dilution with every new batch of anti-DIG-HRP. We have learned that use of 1:1000 to 1:2000 dilution of the anti-DIG-HRP antibody yields good results to decrease background staining. Alternatively, the background can be further reduced by preadsorbing the antibody either with zebrafish acetone powder (Jowett, 2001, Zaidi et al, 2000) or with prehybridized zebrafish embryos (Thisse et al, 2004).

Fluorescent RNA and nuclear staining
For HRP-conjugated antibodies (anti-DIG POD, Roche 1 207 733), TSA can be performed with either of the fluorescent labels Cy3 or Cy5 (PerkinElmer NEL 704A and NEL 705A). For reasons of our local CLSM setup, we concentrated on the TSA/Cy3 fluorescence system; the TSA/Cy5 protocol is very similar to the method described in this paper. To visualize the outline of the embryos in relation to the gene expression location, an additional nuclear staining with SYTOX Green (Molecular Probes S-7020) is performed. To make optimal use of the nuclear staining, it should be applied just prior to the CLSM imaging session. Fading can be overcome by storing embryos in an antifading reagent (e.g., Gelvatol- DABCO) before mounting them for microscopy. In an antifading agent, the specimen and the fluorescent signals remain more stable for a longer period, allowing repeated imaging of the same specimen.

FIG. 1. A panel of marker genes expressed in 24 hpf zebrafish embryos. Row **A** depicts the results of the standard AP detection. Row **B** depicts a characteristic optical section from the 3D image obtained with zebraFISH, using TSA/Cy3-SYTOX Green. Row **C** is a projection of that same image stack into one image. Row **D** depicts 3D reconstructions of the expression pattern, the embryo outline, and some surrounding tissues as obtained from the 3D image.
Column **1** shows *myoD* expression in the somites: (**B1, C1**) *myoD* expression detected in an image stack of 64 slices; (**D1**) 3D reconstruction of *myoD* expression in white, yolk extension in green, and embryo outline in blue. Column 1 is visualized in oblique dorsal orientation. Column **2** shows *krox20* expressed in rhombomeres 3 and 5: (**B2, C2**) *krox20* gene expression in an image stack of 13 slices; (**D2**) 3D reconstruction of *krox20* expression in white, third ventricle in cyan, partial eye outline in salmon, and embryo outline in blue. Column 2 is visualized in oblique lateral orientation. Column **3** shows the *pax2.1* expression pattern at the midbrain-hindbrain boundary and the optic stalk: (**B3, C3**) show gene expression detected in an image stack of 97 slices; (**D3**) 3D reconstruction of *pax2.1* gene expression patterns in white, embryo outline in blue, optic cup in salmon. Column 3 is visualized in oblique lateral orientation. Column **4** shows *otx2* expressed in the diencephalon and mesencephalon: the image stack in (**B4, C4**) is 74 slices; (**D4**) 3D reconstruction of *otx2* gene expression in white, embryo outline in blue, optic cup in salmon. Column 4 is visualized in oblique lateral orientation. For all four genes, the pattern generated with zebraFISH corresponds to the pattern generated with AP detection. The 3D reconstructions give insight into the extension of the pattern within the embryo as well as clear spatial relations with a number of anatomical domains. These domains coincide exactly with the domains annotated in the three-dimensional digital atlas of zebrafish development (bio-imaging.liacs.nl).
FIG. 2. Gene expression patterns of *mpx* in 36 hpf and 48 hpf zebrafish embryos with TSA/Cy3 detection. (**A**) At 36 hpf, an image stack of 70 slices. For this image only TSA detection was applied. The *mpx* expressing cells are clearly visible as dispersed over the yolk and also visible in the head; *mpx* expressing cells also accumulate in the ventral venous plexus (not shown). (**B**–**D**) At 48 hpf, image stacks of 78 slices using TSA/Cy3-SYTOX Green detection. Expression is visible in single cells scattered over the yolk and in the head. Characteristic slices show single cell imaging (arrow pointing to *mpx* expressing cell) in the brain (**B**) and yolk sac (**C**). (**D**) A projection of the whole stack showing the pattern of the *mpx* gene at 48 hpf.

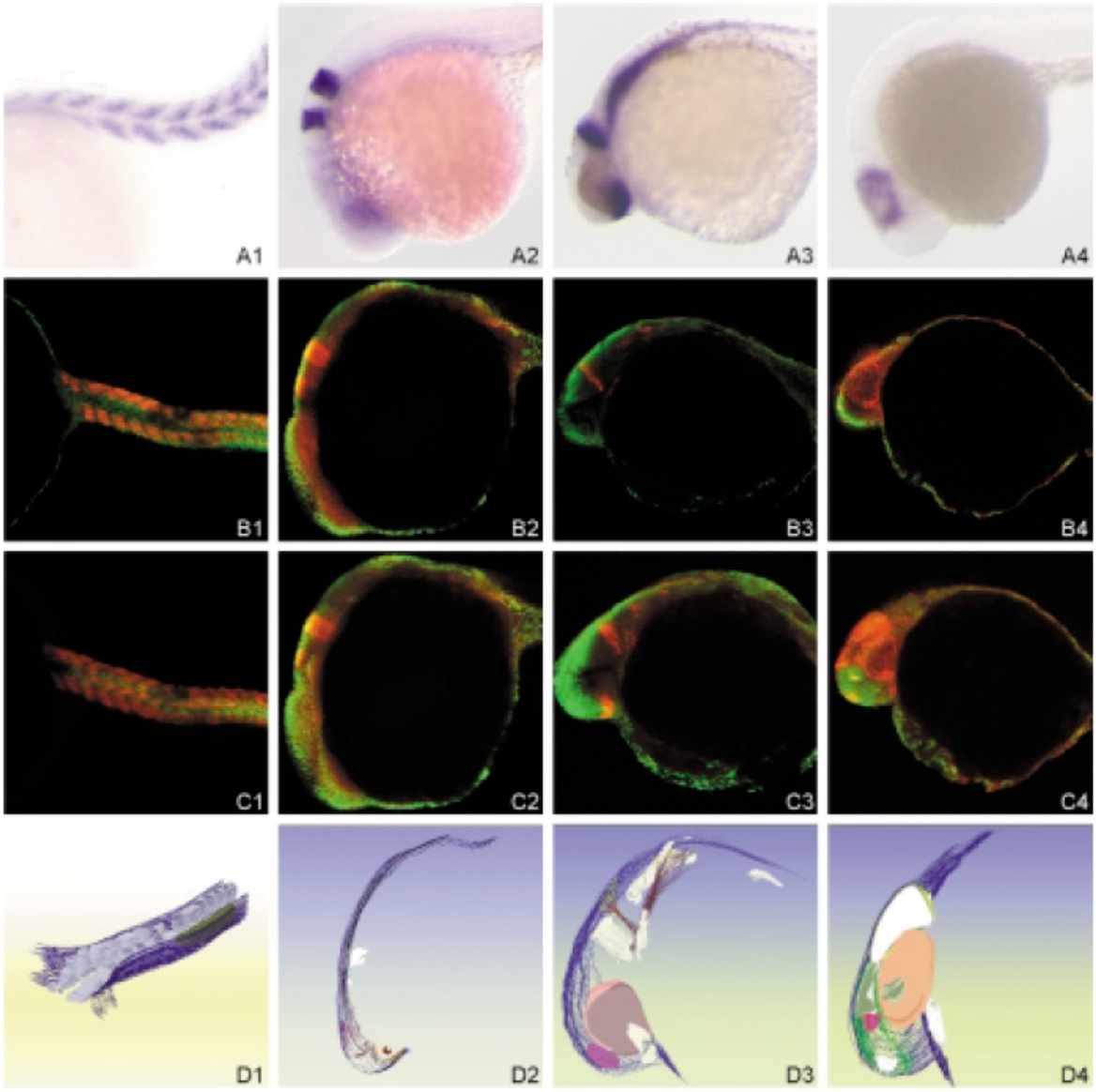

FIG. 1.

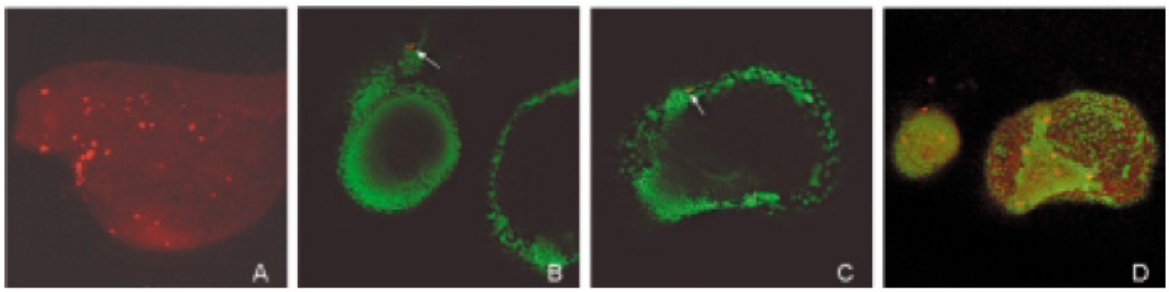

FIG. 2.

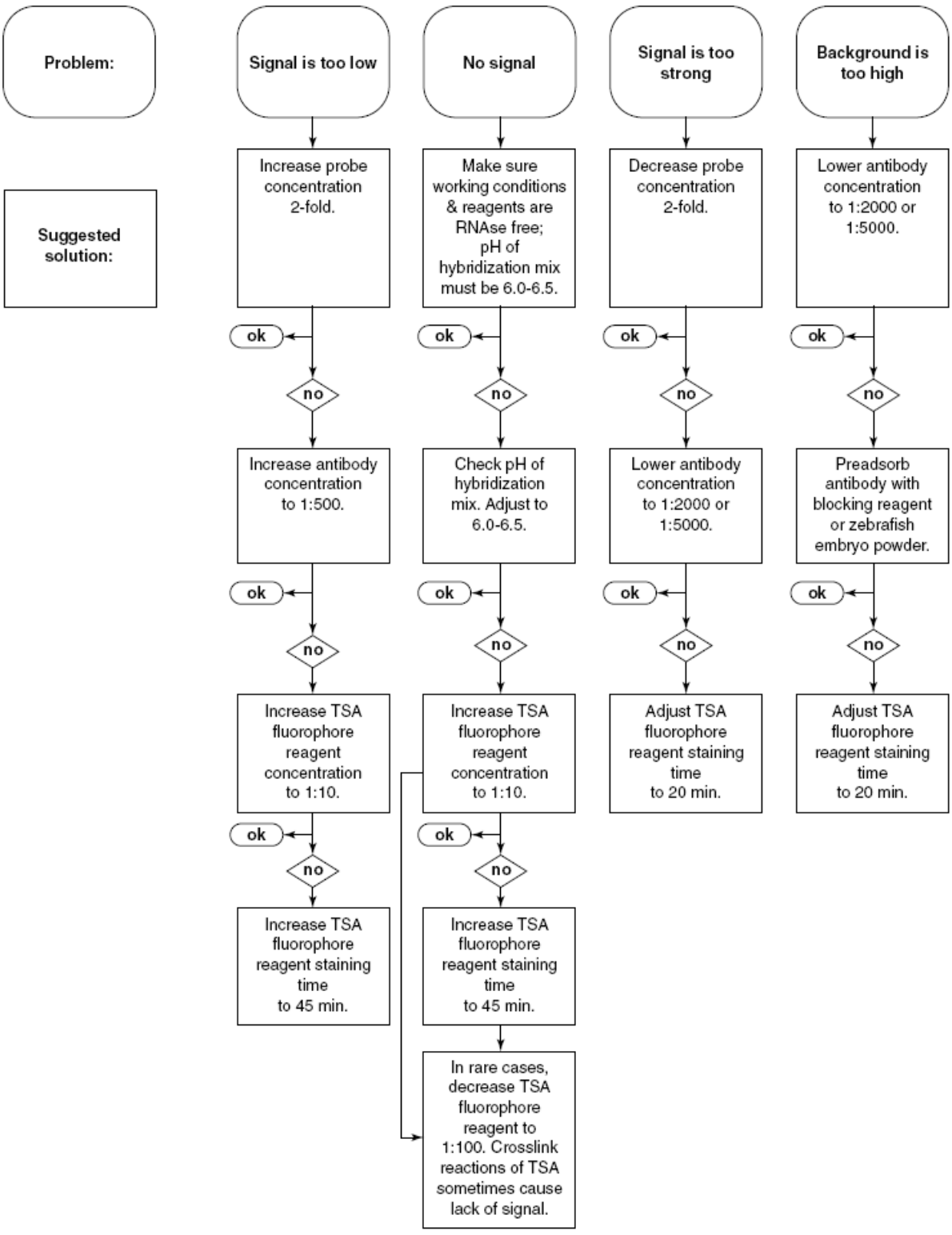

FIG. 3. Schematic summary of possible problems, with flowchart for problem-solving.

Optimization of TSA reaction
In order to obtain optimal results for the amplification reaction we found it necessary to titrate both anti-DIG-HRP antibodies and the TSA fluorophore working solution for every new batch. If adjusting the probe or antibody concentration still results in low signal, the TSA/Cy3 or Cy5 concentration needs to be adjusted; this is also recommended by the manufacturer (personal communication, Grootjans, PerkinElmer Europe). These suggestions for optimization are summarized in Figure 3.

Mounting and storage
The embryos are mounted in glycerol or Gelvatol- DABCO for CLSM on glass bottom dishes (WillCo). After imaging, embryos are stored in Gelvatol-DABCO at 4°C. It should be noted that the glass bottom dishes were selected because an inverted CSLM setup was used.

Control reaction
To check the *in situ* hybridization procedure and the used solutions and reagents, each ISH procedure is performed simultaneously with an NBT/BCIP (Roche) for anti-DIG AP and DAB or BMblue (Roche) for anti-DIG-HRP. Since every probe requires some fine tuning, AP detection gives an indication of the probe concentration to obtain sufficient signal for FISH. Eventually, antibody concentration can be adjusted.

DISCUSSION

In this paper we present a method and protocol for FISH in zebrafish embryos to visualize patterns of gene expression. This protocol is particularly suitable for three-dimensional imaging of zebrafish embryos with confocal laser scanning microscopy.
In order to make FISH applicable for a wide range of probes, signal amplification is used. In parallel with the protocol for FISH, a methodology for image processing of the CLSM images is being developed. Processing these images allows analytical approaches to patterns of gene expression which can be assisted by our three-dimensional atlas of zebrafish development.
The FISH protocol based on the TSA fluorescence system yields clear, strong, specific, and localized expression patterns with a low background. The staining procedure and time is short (30 minutes). In combination with the protocol, we have provided a range of solutions to possible problems. In addition to our findings on reducing background staining, Clay and Ramakrishnan (2005) suggest using Western blocking solution (Roche) and have shown good results with that reagent.
The FISH protocol described in this paper is adapted from the protocol described by Thisse (1993, 2004) with modifications, especially for the fluorochrome tagging and staining. The combination of the SYTOX nuclear staining with TSA/Cy3 or Cy5 detection provides an excellent pair of fluorescent markers to discern gene expression patterns, the outline of the embryo, and the outline of anatomical structures. An alternative to the TSA/Cy3 or Cy5 method based on the AP detection protocol described by Thisse et al (1993, 2004) is enzyme-linked fluorescence signal detection (ELF, Molecular Probes),which also produces a strong signal. For the ELF approach, the staining reaction needs to be monitored. Moreover, it requires a longer staining time,

which can result in higher background. ELF signal detection therefore is a possible alternative but produces less specifically localized precipitate.
Using CLSM imaging, three-dimensional images from the gene expression patterns are produced in a nondestructive manner. Application of the zebraFISH protocol for a range of zebrafish marker genes will result in collections of three-dimensional images of gene expression patterns. Our goal is to make these patterns comparable in a quantitative manner. In some cases, comparing gene expression patterns can be realized using multiple channels in the CLSM. This is restricted by the laser lines available, and thus different for each CLSM. Therefore, we argue that patterns should be compared on an individual basis as well as to a standard. For developmental genetics, a whole range of marker genes and their three-dimensional patterns should be made available in an internet repository, so that researchers can appreciate each pattern separately and combine such results with their own findings.
The results presented in this paper concern embryos that are opaque; using CLSM, a certain depth penetration can be realized, but for older embryos and larval stages, this is difficult. We therefore propose to handle these embryos and larvae with multiphoton laser microscopy, which has a considerable higher potential for depth penetration in the specimen.
In our research we are in the process of providing the necessary computerized tools to make comparisons of spatial patterns possible through digital three-dimensional images and models. We have developed a general three-dimensional reference system for the developing zebrafish embryo, the three-dimensional digital system is used to project the three-dimensional patterns of gene expression that are obtained from application of zebraFISH. A database for these images has been developed: the gene expression database (Bei et al, 2006; Verbeek et al, 2002) directly relates to the three-dimensional atlas of zebrafish development (bio-imaging.liacs.nl). Dedicated tools for the mapping of these CLSM images have also been developed (Verbeek et al, 2004). Three-dimensional visualizations of the patterns of gene expression can provide additional insight into the spatial distribution of gene expression. We have shown this with the three-dimensional reconstructions we have made from the sample FISH images. Different modes of visualization further help in the understanding of complex patterns. The three-dimensional models underlying these visualizations are stored with the three dimensional images.
In the near future, zebraFISH will also be used in combination with transgenic GFP-lines and immunostaining to analyze colocalization (Manders et al, 1993). Our prime focus remains on generating three-dimensional patterns for a gene expression database.

ACKNOWLEDGMENTS

This work is partially supported by The Netherlands Research Council through the Bio-Molecular Informatics Programme of Chemical Sciences, and the ZF-Models programme supported through the European Community Sixth Framework Programme (grant LSHGCT- 2003-503496). We would like to thank Marjo den Broeder, who worked with F.J. Verbeek on FISH while he was at the Hubrecht Laboratory; Dr. Jeroen Bakkers, who kindly provided us with probes; and Laura Bertens for her contributions on the *mpx in situ* hybridizations. We also thank Dr. J. Grootjans (PerkinElmer Europe, Brussels) for his valuable advice, and Peter Hock for his assistance with the preparation of the figures.

APPENDIX

Fluorescent *in situ* hybridization (zebraFISH) protocol

Note: Up to the first post-hybridization wash step, all work should be carried out under RNAse free conditions. Use gloves, sterile disposable tubes, pipette tips and transfer pipettes, and sterilized glassware. Autoclave buffers for 20 min at 120°C. Nuclease-free Tween 20, proteinase K, tRNA, and heparin are added after autoclaving.

1. Embryo fixation

Dechorionate embryos and fix overnight in 4% paraformaldehyde in PBS at 4°C.
Wash 3 times for 5 min in PBST at room temperature.
Dehydrate through methanol series: 5 min 25%, 10 min 50%, 5 min 75% methanol in PBST, wash twice in 100% methanol.
Place embryos in 100% methanol for at least 24 h at -20°C or store at -20°C up to several months.

2. Pretreatment and prehybridization

Inhibit endogenous peroxidase activity in 3% H_2O_2 in methanol for 20 min at room temperature.
Rehydrate embryos through methanol series: 5 min 75%, 10 min 50%, 5 min 25% methanol in PBST.
Wash 4 times for 5 min in PBST at room temperature.
Permeabilize the embryos by digestion with 10 ug/mL proteinase K (Promega V3021) at 37°C.
Duration depends on developmental stage:
Blastula, gastrula, and somitogenesis (<18 somites) stages 30 sec to 1 min
24 hpf stage up to 10 min
Embryos older than 24 hpf 20–30 min
Wash in PBST for 5 min.
Refix embryos in 4% paraformaldehyde in PBST for 20 min at room temperature.
Wash 5 times for 5 min in PBST at room temperature.
Prehybridize in hybridization buffer pH 6.0–6.5 for 2–5 h at 55°C.
Continue directly with hybridization, or store embryos in hybridization buffer at -20°C up to several weeks.

3. Hybridization

Remove the hybridization buffer and discard.
Replace with fresh hybridization buffer containing 100 ng of digoxigenin-labeled antisense riboprobe.
Hybridize overnight at 55°C.

4. Post-hybridization washes

Remove probe mix from embryos and store at -20°C. Probes can be reused up to 3 times.
Wash briefly in hybridization buffer without tRNA and heparin.
In the following wash steps, tRNA and heparin are also omitted from the buffer.
Bring embryos to 2x SSC environment by successive 15 min wash steps at 55°C over the following
gradient:
75% hybridization buffer/25% 2x SSC
50% hybridization buffer/50% 2x SSC
25% hybridization buffer/75% 2x SSC
100% 2x SSC.
Wash 2 times for 30 min in 0.2x SSC at room temperature.
Bring embryos to PBST environment by successive 10 min wash steps at room temperature over

the following gradient:
75% 0.2x SSC/25% PBST
50% 0.2x SSC/50% PBST
25% 0.2x SSC/75% PBST
100% PBST.

5. Antibody incubation and blocking reaction
Incubate embryos for 2–5 h at room temperature in antibody buffer with slow agitation.
Meanwhile, dilute DIG POD (Roche 1207733) for anti-DIG-HRP detection 1:1000 in antibody buffer and preadsorb for 2 h at room temperature with slow agitation.
Optional: Preadsorb anti-DIG-POD in zebrafish acetone powder as described by Jowett (2001), or with a batch of prehybridized embryos as described by Thisse (2004) with Western blocking reagent (Roche 11921673001).(Clay and Ramakrishnan, 2005)
Remove antibody buffer with the preadsorbed anti-DIG-POD antibody solution and incubate overnight with slow agitation at 4°C.

6. Post-antibody washes and detection with fluorescent dyes
Remove POD-conjugated antibody solution from embryos.
Wash 6 times for 15 minutes in PBST at room temperature.
Prepare TSA/Cy3 or TSA/Cy5 substrate according to instructions of the manufacturer (PerkinElmer): dilute Cy3/Cy5 reagent 1:50 in the amplification buffer supplied by manufacturer.
Incubate at room temperature in the dark for 30 minutes.
Wash 8 times for 15 minutes in PBST.

7. Nuclear staining with SYTOX Green
Preferably perform just prior to microscopy, as the staining will diminish over time. Stain with 100 nM SYTOX Green (Invitrogen-Molecular Probes, S-7020) for 1 h at room temperature.
Wash 6 times for 15 min in PBST.

8. Mounting for microscopy
Mount embryos in Gelvatol containing 100mg/ml DABCO (1,4-diazabicyclo[2.2.2]octane) in a glass-bottom dish (Willco Wells).
Preserve embryos in Gelvatol DABCO at 4°C in the dark. Storing in Gelvatol DABCO preserves nuclear staining up to 3 weeks.

Solutions
PBS 10x stock solution: 75.97g NaCl, 12.46g $NaH_2PO_4 \cdot 2H_2O$, 4.80g $Na_2HPO_4 \cdot H_2O$ in 1 L of milliQ water. Adjust pH to 7.0.
PBST: PBS + 0.1% Tween 20
SSC (20x stock solution: 3M NaCl, 300 mM trisodium citrate)
1 M citric acid
Hybridization buffer: 50% deinonized formamide (Sigma F9037), 5x SSC, 0.1% Tween 20, pH adjusted to 6–6.5 with 1M citric acid, 50 ug/ml heparin (Sigma H3393), 500 ug/ml tRNA (Sigma R7876) (Thisse et al. 2004)
Antibody buffer: PBST containing 2% sheep serum (Sigma S-2263) and 2 mg/ml BSA
Gelvatol containing 100mg/ml DABCO (1,4-diazabicyclo[2.2.2]octane)

Additional remarks to the ZebraFISH protocol

4:**To improve background reduction: replace PBST by TBST for the following gradient**:

Bring embryos to **TBST** environment by successive 10 min wash steps at room temperature over the following gradient:

75% 0.2x SSC/ 25% **TBST**
50% 0.2x SSC/ 50% **TBST**
25% 0.2x SSC/ 75% **TBST**
100% **TBST**

And 5: **Replace PBST by TBST for the Antibody buffer:**
- Dilute DIG POD, Roche 1207733 for anti DIG- Horse Radish Peroxidase detection 1: 1000 in antibody buffer Antibody buffer : **TBST** containing 2% sheep serum (Sigma., cat. nr. S-2263), and 2 mg/ ml BSA.

6: To improve background reduction during post-antibody washes and detection with fluorescent dyes:
- Replace PBST by TBST at room temperature.
- Optional: wash 6 x 1 hr at room temperature,
- Optional: wash overnight at +4° to obtain higher background reduction.

To improve signal of weak or short probes (< 450 bp): use TSA™ Plus Cyanine 3 System
(NEL744, Perkin Elmer).
Dissolve TSA Plus - Cy3 / Cy5 reagent in 150 ul DMSO (molecular grade).
Dilute Cy3 / Cy5 reagent 1: 50 in the amplification buffer supplied by manufacturer.

7. Nuclear staining with SYTOX Green:
Stain with 100 nM SYTOX Green (Invitrogen-Molecular Probes, S-7020) in **TBST**. Add 0.01 % Triton X.
- Stain for at least 1 h at room temperature
- Wash 6 x 15 min in **TBST**.
- Optional: stain overnight at +4°.

Solutions:

1xTBS:
6.05 g Tris (50mM)
8.76 g NaCl (150 mM) in 1 liter of milliQ water. Adjust pH to 7.5.
TBST: TBS + 0.1 % Tween 20

SYTOX Green (Invitrogen-Molecular Probes, S-7020): 100 nm in **TBST;** add 0.01% Triton X-100 (Sigma-Aldrich 234729