ZebraFISH: fluorescent in situ hybridization protocol and three-dimensional imaging of gene expression patterns

Welten, M.C.M.; Haan, S.B. de; Boogert, N. van den; Noordermeer, J.N.; Lamers, G.E.M.; Spaink, H.P.; ... ; Verbeek, F.J.

Note: To cite this publication please use the final published version (if applicable).

ZebraFISH: Fluorescent *In Situ* Hybridization Protocol and Three-Dimensional Imaging of Gene Expression Patterns

MONIQUE C.M. WELTEN,[1,2] SIMON B. DE HAAN,[1,2] NIELS VAN DEN BOOGERT,[1]
JASPRIEN N. NOORDERMEER,[3] GERDA E.M. LAMERS,[2] HERMAN P. SPAINK,[2]
ANNEMARIE H. MEIJER,[2] and FONS J. VERBEEK[1]

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.[1] 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).[2] 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.[2] 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.[3,4] 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.[3]

For analytical approaches, a true three-dimensional spatial representation of the pattern of gene expression is required.[5] Three-dimen-

---

[1]Imaging & BioInformatics, Leiden Institute of Advanced Computer Science, Leiden University, Leiden, The Netherlands.

[2]Institute of Biology, Leiden University, Leiden, The Netherlands.

[3]Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands.

sional 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.[3,4] as a foundation. Whole-mount FISH has been successfully applied in *Drosophila*[6,7] and the protocol used in the *Drosophila* community was taken as the starting point to develop and optimize the fluorescent labeling steps for zebraFISH.

The first steps of our FISH protocol are identical to the standard Thisse[3,4] protocol and it diverges at the point of application of the flu-

orescent 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-labeled 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 a strong expression signal with relatively low background staining.[8] 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.[9] The focus of their protocol was on multiplex detection of genes with overlapping expression patterns, with AlexaFluor conjugates used to demonstrate colocalization ex-

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.
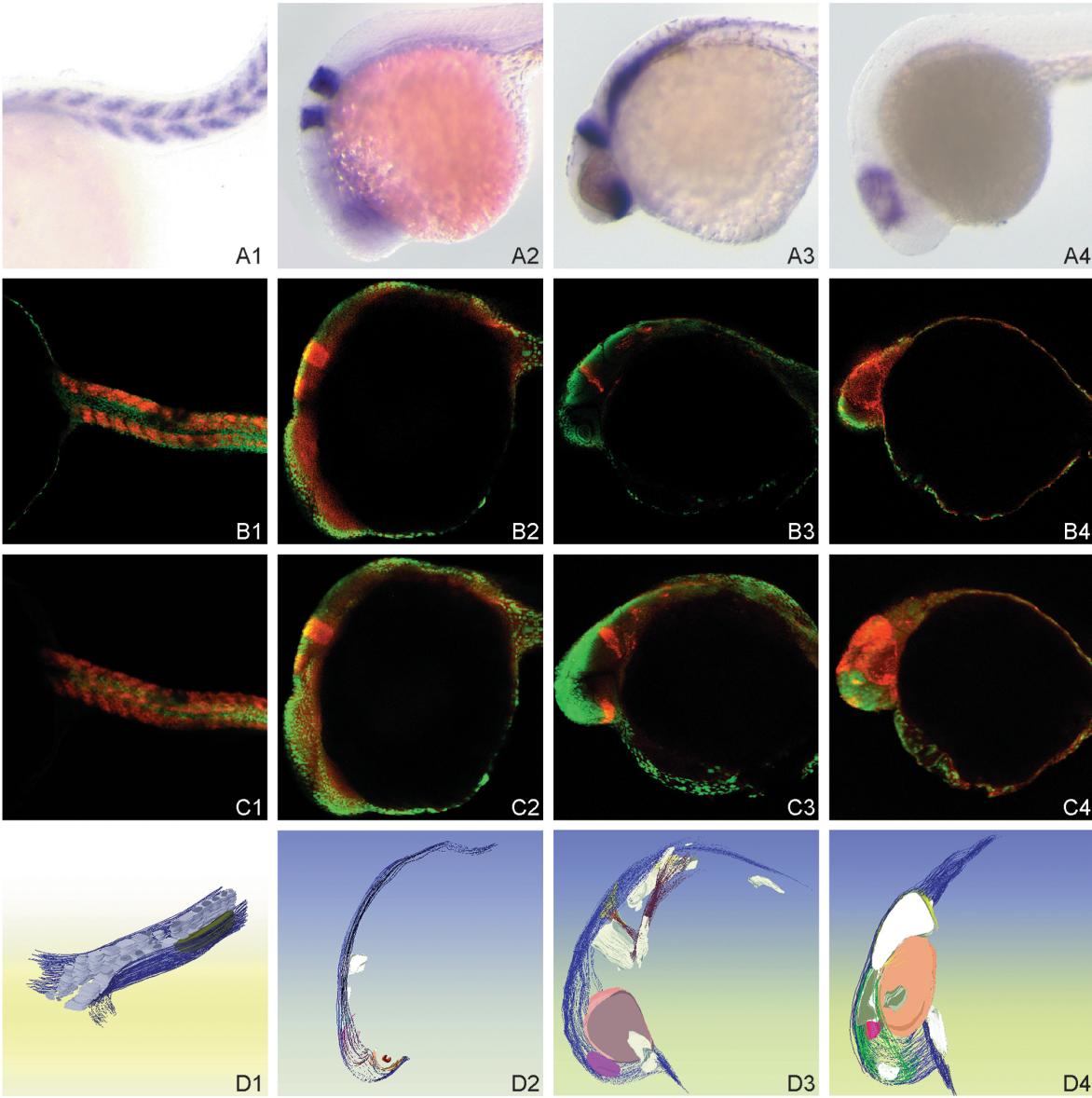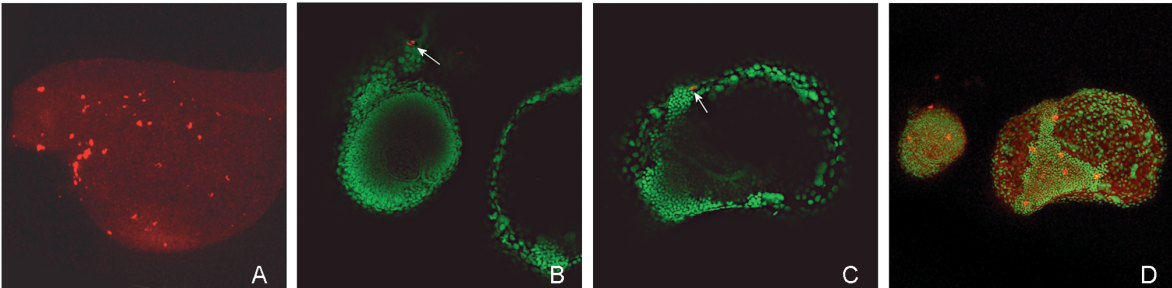
FIG. 1.

FIG. 2.

pression 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.*

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,[10] *krox20* is expressed in rhombomeres 3 and 5,[11] *otx2* is expressed in the diencephalon and mesencephalon,[12] and *pax2.1* shows expression domains in the midbrain-hindbrain boundary, the optic stalk, and the otic vesicle.[13]

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.[14,15] 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.[16] 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 (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.[4,8,9] 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. suggest hybridization at 70°C.[3,4]

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.[17]

Alternatively, the background can be further reduced by preadsorbing the antibody either with zebrafish acetone powder[8,17] or with pre-hybridized zebrafish embryos.[4]

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.

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 fluorescent *in situ* hybridization procedure and the used solutions and reagents, each 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[9] 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
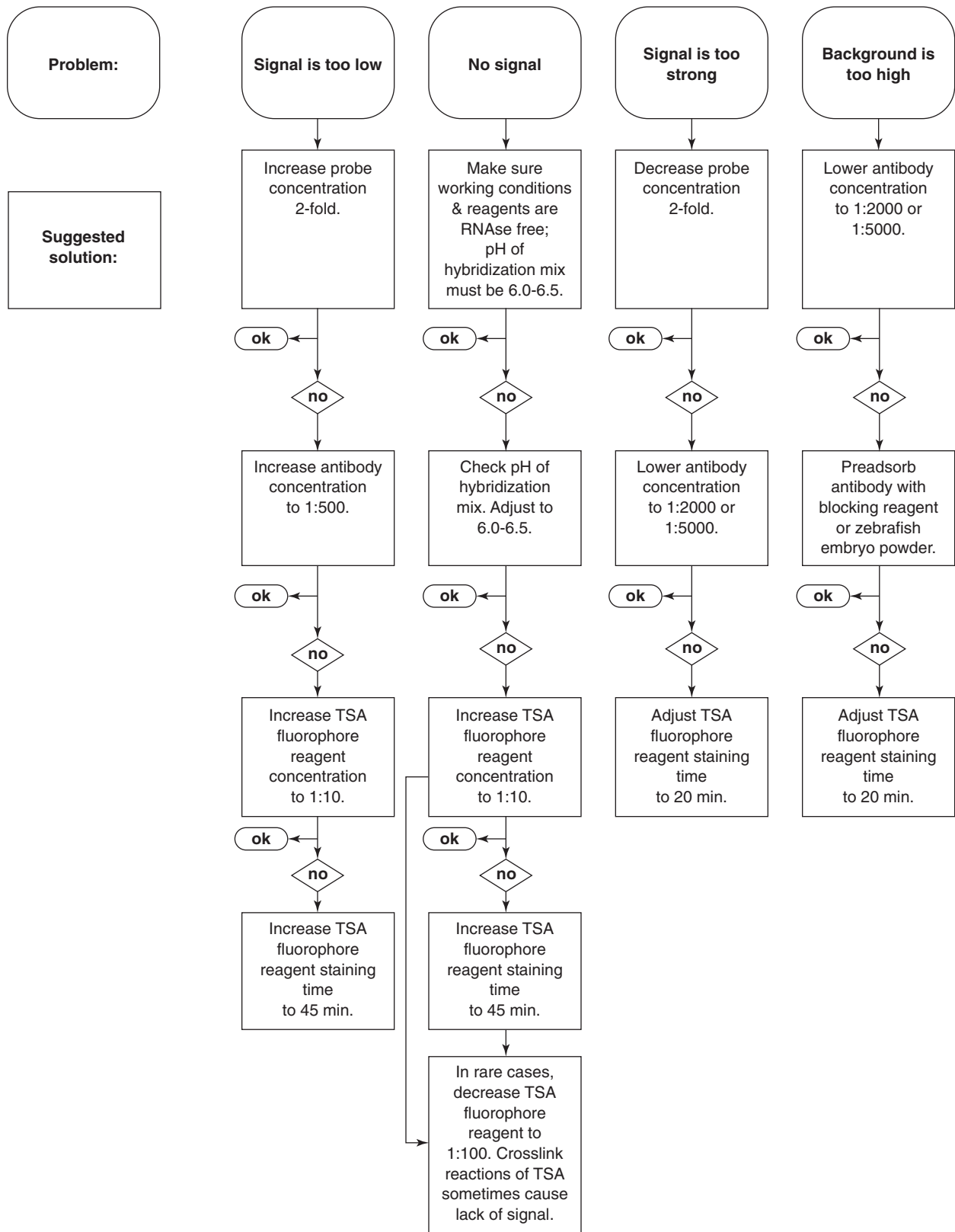
Problem:	Signal is too low	No signal	Signal is too strong	Background is too high

Suggested solution:

Signal is too low
- Increase probe concentration 2-fold.
- ok / no
- Increase antibody concentration to 1:500.
- ok / no
- Increase TSA fluorophore reagent concentration to 1:10.
- ok / no
- Increase TSA fluorophore reagent staining time to 45 min.

No signal
- Make sure working conditions & reagents are RNAse free; pH of hybridization mix must be 6.0-6.5.
- ok / no
- Check pH of hybridization mix. Adjust to 6.0-6.5.
- ok / no
- Increase TSA fluorophore reagent concentration to 1:10.
- ok / no
- Increase TSA fluorophore reagent staining time to 45 min.
- In rare cases, decrease TSA fluorophore reagent to 1:100. Crosslink reactions of TSA sometimes cause lack of signal.

Signal is too strong
- Decrease probe concentration 2-fold.
- ok / no
- Lower antibody concentration to 1:2000 or 1:5000.
- ok / no
- Adjust TSA fluorophore reagent staining time to 20 min.

Background is too high
- Lower antibody concentration to 1:2000 or 1:5000.
- ok / no
- Preadsorb antibody with blocking reagent or zebrafish embryo powder.
- ok / no
- Adjust TSA fluorophore reagent staining time to 20 min.

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

Thisse[3,4] 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.[3,4] 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.[14] 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 one researcher 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 atlas of zebrafish development.[5,15] This reference 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[19,20] 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.[18] 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.[22] Our prime focus remains on generating three-dimensional patterns for a gene expression database.

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.[21]

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, 5× SSC (20× SSC = 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-labeled 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 2× SSC (75%/25%, 50%/50%, 25%/75%) to a final wash in 100% 2× SSC. Subsequently, the embryos were washed 2 times in 0.2× SSC for 30 min at room temperature and washed in 10-min steps at room temperature

over a gradient of 0.2× SSC 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 preincubation, 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.[3,4] was carried out in parallel. Each control ISH was examined on a stereo microscope and photographed with a digital camera for later reference.

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

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. In our setup, excitation resulted in a green and a red channel. All images shown were obtained with a 10× 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 × 1024 pixels. The CLSM images are saved as two-channel multiple TIFF files and these files were processed with dedicated image processing software.[19] The three-dimensional reconstructions (see Fig. 1) were produced from the CLSM images using the TDR-3Dbase annotation and reconstruction software.[14,15] 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.

REFERENCES

1. Stern HM, Zon LI. Cancer genetics and drug discovery in the zebrafish. Nat Rev Cancer 2003;3:533–539.
2. Wilkinson DG. *In situ* hybridization, a practical approach. Oxford University Press, Oxford, England, 1998.
3. Thisse C, Thisse B, Schilling TF, Postlethwait JH. Structure of the zebrafish *snail1* gene and its expression in wild-type, *spadetail* and *no tail* mutant embryos. Development 1993;119:1203–1215.
4. Thisse B, Heyer V, Lux A, Alunni V, Degrave A, Seiliez I, et al. Spatial and temporal expression of the zebrafish genome by large scale *in situ* hybridization screening. Methods Cell Biol 2004;77:505–519.
5. Verbeek FJ, Lawson KA, Bard JB. Developmental bioinformatics: linking genetic data to virtual embryos. Int J Dev Biol 1999;43:761–771.
6. Kearny JB, Wheeler SR, Estes P, Parente B, Crews ST. Gene expression profiling of the developing *Drosophila* CNS midline cells. Dev Biol 2004;275:473–492.
7. Paddock SW. An introduction to confocal imaging. In: Confocal Microscopy: Methods and Protocols. Totowa, NJ: Humana Press, 1999:1–34.
8. Zaidi AU, Enomoto H, Milbrandt J, Roth KA. Dual fluorescent in situ hybridization and immunohistochemical detection with tyramide signal amplification. J Histochem Cytochem 2000;48:1369–1375.
9. Clay H, Ramakrishnan L. Multiplex fluorescent *in situ* hybridization in zebrafish embryos using tyramide signal amplification. Zebrafish 2005;2:105–111.
10. Weinberg ES, Allende ML, Kelly CS, Abdelhamid A, Murakami T, Andermann P, et al. Developmental regulation of zebrafish *MyoD* in wild type, *no tail* and *spadetail* embryos. Development 1996;122:271–280.
11. Woo K, Fraser SE. Specification of the hindbrain fate in the zebrafish. Dev Biol 1998;197:283–296.
12. Mercier P, Simeone A, Cotelli F, Boncinelli E. Expression pattern of two *otx* genes suggest a role in specifying anterior body structures in zebrafish. Int J Dev Biol 1995;39:559–573.
13. Lun K, Brand M. A series of no isthmus (noi) alleles of the zebrafish *pax2.1* gene reveals multiple signaling events in development of the midbrain-hindbrain boundary. Development 1998;125:3049–3062.
14. Verbeek FJ, Den Broeder MJ, Boon PJ, Buitendijk B, Doerry E, Van Raaij EJ, Zivkovic D. A standard atlas of zebrafish embryonic development for projection of experimental data. Proc SPIE Internet Imaging I 2000;3964:242–252.
15. Verbeek FJ. Theory and practice of 3D reconstructions from serial sections. In: Baldock RA, Graham J (eds.) Image Processing, A Practical Approach. Oxford: Oxford University Press, 2000:153–195.
16. Lieschke GJ, Oates AC, Crowhurst MO, Ward AC, Layton JE. Morphologic and functional characterization of granulocytes and macrophages in embryonic and adult zebrafish. Blood 2001;98:3087–3096.
17. Jowett T. Double *in situ* hybridization techniques in zebrafish. Methods 2001;23:345–358.

18. Verbeek FJ, Rodrigues DD, Spaink HP, Siebes A. Data submission of 3D image sets to a bio-molecular database using active shape models and a 3D reference model for projection. Proc SPIE Internet Imaging V 2004:5304:13–23.

19. Bei Y, Belmamoune M, Verbeek FJ. Ontology and image semantics in multimodal imaging: submission and retrieval. Proc SPIE Internet Imaging VII 2006; 6061:C1–C12.

20. Verbeek FJ, Boon PJ, Sloetjes H, Van der Velde R, Vos N. Visualization of complex data sets over Internet: 2D and 3D visualisation of the 3D digital atlas of zebrafish development. Proc SPIE Internet Imaging III 2002;4672:20–29.

21. Kimmel CB, Ballard WW, Kimmel SR, Ullman B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn 1995;203:253–310.

22. Manders EMM, Verbeek FJ, Aten JA. Measurement of co-localisation of objects in dual colour confocal images. J.Microscopy 1993;169:375–382.

Address reprint requests to:
Fons J. Verbeek
Imaging & BioInformatics
Leiden Institute of Advanced Computer Science
Leiden University
Niels Bohrweg 1
2333 CA, Leiden
The Netherlands

E-mail: fverbeek@liacs.nl

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 2× SSC environment by successive 15 min wash steps at 55°C over the following gradient:

75% hybridization buffer/25% 2× SSC

50% hybridization buffer/50% 2× SSC

25% hybridization buffer/75% 2× SSC

100% 2× SSC.

Wash 2 times for 30 min in 0.2× SSC at room temperature.

Bring embryos to PBST environment by successive 10 min wash steps at room temperature over the following gradient:
75% 0.2× SSC/25% PBST
50% 0.2× SSC/50% PBST
25% 0.2× 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,[17] or with a batch of prehybridized embryos as described by Thisse[4] with Western blocking reagent (Roche 11921673001).[9]

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 10× 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 (20× stock solution: 3M NaCl, 300 mM trisodium citrate)

1 M citric acid

Hybridization buffer: 50% deinonized formamide (Sigma F9037), 5× 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)[4]

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)