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# In Situ Hybridization for Sipunculus nudus Coelomic Fluid

--Manuscript Draft--

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Dear Editor,

We really appreciate your comments on our video submitted for publication in Journal of Visualized Experiments (JoVE61022). We have carefully edited every point stated by the editors or reviewers.

We hope that our revised video is suitable for publication. For we are not so professional with video production and modification, there might be still some issues in our video. If it is possible, we can pay for the work of video improvement.

If our work could be accepted for publication in Journal of Visualized Experiments, I wish it could be “in-press” a little earlier. So that it might be read by more people and would be helpful for *Sipunculus nudus* study.

Many thanks again for your thoughtfulness and look forward to hearing from you.

Best wishes,

Wenhua Li

**TITLE:**

# In situ Hybridization for Sipunculus nudus Coelomic Fluid

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**KEYWORDS:**

RNA Localization, Digoxigenin, Antisense Riboprobe, Sense Riboprobe, Expression Pattern, Marine Worm

**SUMMARY:**

This protocol describes an effective in situ hybridization approach to detect the mRNA expression levels and spatial patterns of target genes in *Sipunculus nudus* coelomic fluid.

**ABSTRACT:**

In situ hybridization (ISH) is a very informative technique to present cellular distribution patterns of specific genes (e.g., mRNA and ncRNA) in tissues. The sipunculid worm *Sipunculus nudus* is a crucial fishery resource as it has high nutritional and medicinal values. Currently, the research on the molecular biology of *Sipunculus nudus* is still in its infancy. The purpose of this article is to develop a sensitive method for localizing specific mRNA in *Sipunculus nudus* coelomic fluid. The protocol includes detailed steps of ISH, including digoxigenin-labeled antisense and sense riboprobe preparation, coelomic fluid collection and section preparation, specific riboprobe hybridization, antibody incubation, coloration and post-coloration treatments. The representative results obtained from a successful experiment using this method are demonstrated. The protocol should be applicable to other Sipuncula species as well.

**INTRODUCTION:**

ISH, using a labeled nucleic acid probe to detect the specific DNA or RNA sequence of interest, is a useful method to describe the spatial expression pattern of target genes in morphologically preserved tissues[1-3]. Normally, the target sequence is generated by polymerase chain reaction (PCR), and then used as the template to synthesize the antisense/sense RNA probe labeled with digoxigenin uridine-5'-triphosphate. Samples are fixed and permeabilized before incubation with riboprobe. After washing off the excess probe, hybridization is visualized by immunohistochemistry using an anti-digoxygenin antibody, which is alkaline phosphatase-conjugated[3-6].

*Sipunculus nudus* (Phylum Sipuncula; order Sipunculida: Sipunculidae) is an unsegmented, coelomate and bilaterally symmetrical marine worm[7,8]. *Sipunculus nudus* is a cosmopolitan species widely distributed in tropical and temperate coastal waters. It is also an important marine fishery resource in southern China because of its high nutritional and medicinal values[9,10]. However, *Sipunculus nudus* in molecular biology is still in its infancy. To fully understand the biological role of genes, the investigation of genes expression patterns at a cellular resolution is of great interest. In the non-model organism *Sipunculus nudus*, the ISH method, which is an ideal method to detect the genes expression patterns, has not yet been established. Its coelomic fluid contains several cell types, including granulocytes, urn cells, vesicular cells, germ cells, erythrocytes, etc[11]. The double sex/mab-3 related transcription factor-1 (*dmrt1*), used as a representative gene in this method, is a highly conserved transcriptional regulator of sex determination and differentiation in most species ranging from invertebrates to mammals[12,13]. In a range of species (i.e., black porgy, etc.)[14], *dmrt1* was expressed in the Sertoli cells, surrounding the germinal cells, whose function is similar to trophoblast cells of *Sipunculus nudus*. Therefore, we hypothesized that *dmrt1* of *Sipunculus nudus* is expressed in trophoblast cells of spermatozeugmata, and the result of the ISH method clearly confirmed the hypothesis.

This protocol for the first time describes ISH, with digoxigenin-labeled antisense/sense mRNA probes, to determine mRNA expression patterns in its coelomic fluid smear. The optimal reaction conditions are supplied, which allow very sensitive visualization of mRNA expression at high resolution. The developed ISH method could be potentially applied in more Sipunculida species other than *Sipunculus nudus.*

**PROTOCOL:**

The animal procedure was approved by the Animal Care and Welfare Committee of Huaqiao University.

## 1. Riboprobe preparation

### 1.1. Primer design

1.1.1. Open the program Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/). Copy the *dmrt1* sequence (GenBank: MK182259) into the sequence window.

1.1.2. Set primer length (23-25 bp), melting temperature (55-60 °C), and G/C content (40-60%). Click **Pick Primers**.

NOTE: The minimal size required for the RNA probe is approximately 500 bp. Longer probes normally have higher specificity.

1.1.3. Add the T7 RNA polymerase promoter sequence (5′ -TAATACGACTCACTATAGGG-3′) to one of the selected primers. The *dmrt1* primer sequences for ISH are shown in **Table 1**.

NOTE: For sense probes, the T7 RNA polymerase promoter is located at the 5’ end of the forward primers. For antisense probes, the T7 RNA polymerase promoter is located at the 5’-end of the reverse primers.

1.2. PCR amplification

1.2.1. Place the microfuge tubes on ice, and prepare the following reaction mix (for a 50 µL reaction): 1x Taq DNA polymerase mix, 1 µg of cDNA (which is prepared from 100 µL of coelomic fluid), 1 µM forward primer, 1 µM reverse primer and nuclease-free water.

1.2.2. Mix the PCR reaction by pipetting and centrifuge briefly.

1.2.3. Place the reaction tube in a thermal cycler, and run the PCR using the following conditions: initial denaturation at 95 °C for 2 min followed by 36 cycles of denaturation at 95 °C for 30 s, annealing at 55-60 °C for 30 s, extension at 72 °C for 30 s and a final extension at 72 °C for 7 min.

NOTE: The annealing temperature should be optimized according to the primers.

1.3. PCR product purification and verification

1.3.1. Load the 50 µL of PCR mixture directly on a 1% agarose gel. Run the agarose gel at 150-180 V for 10 min in 0.5x TBE. Isolate the specific DNA fragments from the 1% agarose gel.

NOTE: DNA should appear as a single band and not as a smear.

1.3.2. Purify the DNA fragments using a gel extraction kit according to the manufacturer's protocol. Quantify the purified products by spectrophotometry at a wavelength of 260 nm.

1.3.3. Verify the authenticity of the PCR products by sequencing.

NOTE: (Pause point) The purified PCR products can be stored at -20 °C for several months.

1.4. Riboprobe synthesis

1.4.1. Place the RNase-free microfuge tubes on ice, and add the following to the microfuge tube (for a 10 µL reaction): 1x Digoxygenin RNA Labeling Mix, 1x transcription buffer, 0.5 µL of RNase inhibitors, 1 µL of T7 RNA polymerases, 1 µg of PCR product and RNase-free water. Mix the reaction by pipetting and centrifuge briefly. Incubate for 2 h at 37 °C.

1.4.2. Add 2 µL of RNase-free DNase I. Mix the reaction by pipetting and centrifuge briefly. Incubate for 15 min at 37 °C.

1.4.3. Add 2 µL 0.2 M EDTA (pH 8.0). Mix the reaction by pipetting and centrifuge briefly.

1.4.4. Add 2.5 µL of 4 M LiCl and 75 µL of prechilled ethanol to the above reaction. Mix well. Leave for 30 min at -70 °C.

1.4.5. Centrifuge at 12,000 x *g* for 10 min at 4 °C. Decant the ethanol. Add 1 mL of prechilled 70% ethanol (v/v) and wash the precipitation by mixing gently.

1.4.6. Centrifuge at 12,000 x *g* for 5 min at 4 °C. Decant the 70% ethanol and dry the precipitation briefly near an alcohol lamp. Dissolve the precipitation by adding 30 µL of RNase-free water and mix gently.

1.4.7. Load 2 µL of synthesized RNA on a 1% agarose gel. Run the agarose gel at 180 V for 5-10 min in 0.5x TBE. Measure the concentration of the labeled RNA using a spectrophotometer at a wavelength of 260 nm.

1.4.7.1. Use RNase-free microfuge tubes and filter tips to avoid RNase contamination.

NOTE: (Pause point) The digoxygenin-labeled probes can be stored at -70 °C for several months.

## 2. Coelomic fluid collection

2.1. Fix the *S. nudus* on the dissection table with pins. Open the body of the *S. nudus* with small autoclaved scissors. Isolate the coelomic fluid with a pipette.

2.2. Collect and transfer 1 mL coelomic fluid with a pipette to poly-D-lysine treated microscope slides. Apply the coelomic fluid evenly with a pipette tip. Air-dry the slides for 1 h at 37 °C.

## 3. In situ hybridization

3.1. Day 1

3.1.1. Rehydrate the slides in a slide staining jar containing 100 mL of 1x diethyl pyrocarbonate treated phosphate buffered saline (DEPC-PBS). Rehydrate 3 times with 1x DEPC-PBS, 5 min per wash with gentle agitation.

3.1.2. Permeabilize the smear of coelomic fluid by digestion with 10 µg/mL proteinase K at room temperature for 5 min. Incubate the slides in 4% paraformaldehyde (PFA) for 20 min to stop the digestion. Wash the slides in 1x DEPC-PBS with gentle agitation for 5 min 3 times.

3.1.3. Add 50 µL of hybridization mix (HM) containing the sense/antisense riboprobe on the slides and add a cover slip.

3.1.4. Add wet box buffer into the wet box. Put the slides into the wet box and seal well with

paraffin film. Hybridize overnight (at least 16 h) at 60 °C.

3.2. Day 2

3.2.1. Preheat the wash buffer at 65 °C. Immerse the slide in the slide staining jar containing the wash buffer and let it stand until the coverslip slides off automatically. It takes about 5 min.

3.2.2. Wash 2 times with wash buffer at 65 °C, 30 min per wash with gentle agitation. Wash 2 times with 0.2x saline-sodium citrate (SSC) at 65 °C, 30 min per wash with gentle agitation. Wash 2 times with maleic acid buffer containing Tween 20 (MABT) at room temperature, 30 min per wash with gentle agitation.

3.2.3. Incubate the slides at room temperature for 3–4 h in the blocking buffer. Incubate the slides in 1 mL anti-digoxigenin-AP Fab fragments solution diluted at 1/5000 with the blocking buffer at 4 °C overnight.

3.3. Day 3

3.3.1. Remove the antibody solution and wash the slides briefly in MABT. Wash 4 times with MABT at room temperature, 25 min per wash with gentle agitation.

3.3.2. Incubate the slides with alkaline phosphatase buffer at room temperature 3 times, 5 min per wash. Remove the alkaline phosphatase buffer, and add 1 mL of 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) staining solution. Keep the slides in the dark.

3.3.3. Observe the color reaction periodically under an optical microscope. When the color is fully developed (reaction time in the range of 1-4 h), wash the slides 2 times with MABT at room temperature, 5 min per wash with gentle agitation.

3.3.4. Wash 2 times with stop solution at room temperature, 15 min per wash with gentle agitation. Incubate the slides in methanol to remove excess stain, at room temperature for 30 min. According to the background color, the methanol wash can be done 2 or 3 times and the decolorization time can be extended.

3.3.5. Wash 2 times with MABT at room temperature, 5 min per wash with gentle agitation. Transfer the slides to a fresh filter paper. Add 50 µL of glycerol. Add the coverslips and observe microscopically.

NOTE: The solution composition is showed in **Table 2**.

**REPRESENTATIVE RESULTS:**

A summary of the steps involved in ISH is illustrated in **Figure 1**. Antisense and corresponding sense riboprobes for *dmrt1* were synthesized from PCR products amplified from the coelomic fluid cDNAs. The authenticity of the PCR products was verified by direct sequencing. Riboprobes

were synthesized using T7 RNA polymerases according to the manufacturer's protocols and a previous report[4] with some minor modifications. The representative signals of ISH are shown in **Figure 2**. ISH of *Sipunculus nudus* coelomic fluid with antisense riboprobe that targets *dmrt1* reveals purple staining concentrated in trophoblast cells of the spermatozeugmata (**Figure 2A, B**, red arrows). A sense riboprobe was used as a negative control, and the sense riboprobe for *dmrt1* did not detect any hybridization signal (**Figure 2C**).

**FIGURE AND TABLE LEGENDS:**

**Figure 1. Flow diagram of ISH.** Blue boxes are the steps for synthesizing riboprobe. Light green boxes are steps for in situ procedures.

**Figure 2. The expression of *dmrt1* in *Sipunculus nudus* coelomic fluid detected by ISH.** (**A, B**) Hybridization with *dmrt1* antisense riboprobe. (**C**) Hybridization with *dmrt1* sense riboprobe. The red arrows represent hybridization signals in trophoblast cells. sz, spermatozeugmata. Scale bar: 50 μm. This figure has been modified from Li et al.[15].

**Table 1. The *dmrt1* primer sequences.**

**Table 2. The composition of solutions used in the ISH protocol.**

**DISCUSSION:**

Previous studies showed that ISH is suitable for detecting multiple target RNAs[16-18]. In this protocol, we described a high-resolution ISH method to detect the mRNA in coelomic fluid and emphasize the optimized hybridization conditions in *Sipunculus nudus.* The obvious signals of *dmrt1* we observed demonstrated the successful application of this protocol in the detection of gene expression (**Figure 2**).

During the experiment, a series of steps need to be given particular attention. Firstly, sense riboprobes for the target genes must be synthesized as the control. After the synthesis, the quality of the riboprobe should be checked on a gel. Poor RNA synthesis will result in no staining in the sections. Secondly, the sample collection process should be gentle to prevent cell deformation and the experiment must be performed immediately after coelomic fluid collection to prevent RNA degradation. Thirdly, incubation time and times should be exactly followed in all steps without shortening or lengthening. In particular, pay attention to the timing of proteinase K treatment. Too long treatment time of proteinase K will lead to the destruction of the cell structure of the sample, while too short treatment time will not allow the riboprobe to enter the cell properly. Finally, slides should not dry out during the experiment. This method involves a hybridization step at 60 °C, which will increase the risks of reagent evaporation. As stated in the protocol section, slides must be put into a wet box and covered with paraffin film to avoid evaporation.

One limitation of this protocol is the acquisition of riboprobe sequences in non-model organism *Sipunculus nudus*, because poorly sequenced mRNA may confer low specificity of the riboprobe. With the development of sequencing technology, more and more high-quality sequences of

*Sipunculus nudus* will be released, which will greatly improve this situation.

**ACKNOWLEDGMENTS:**

This work was supported by the Young Scientists Fund of the National Natural Science Foundation of China (Grant No. 31801034), the Natural Science Foundation of Fujian Province, China (2016J0101), the Scientific Research Funds of Huaqiao University (15BS306) and Postgraduates' Innovative Fund in Scientific Research of Huaqiao University.

**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1



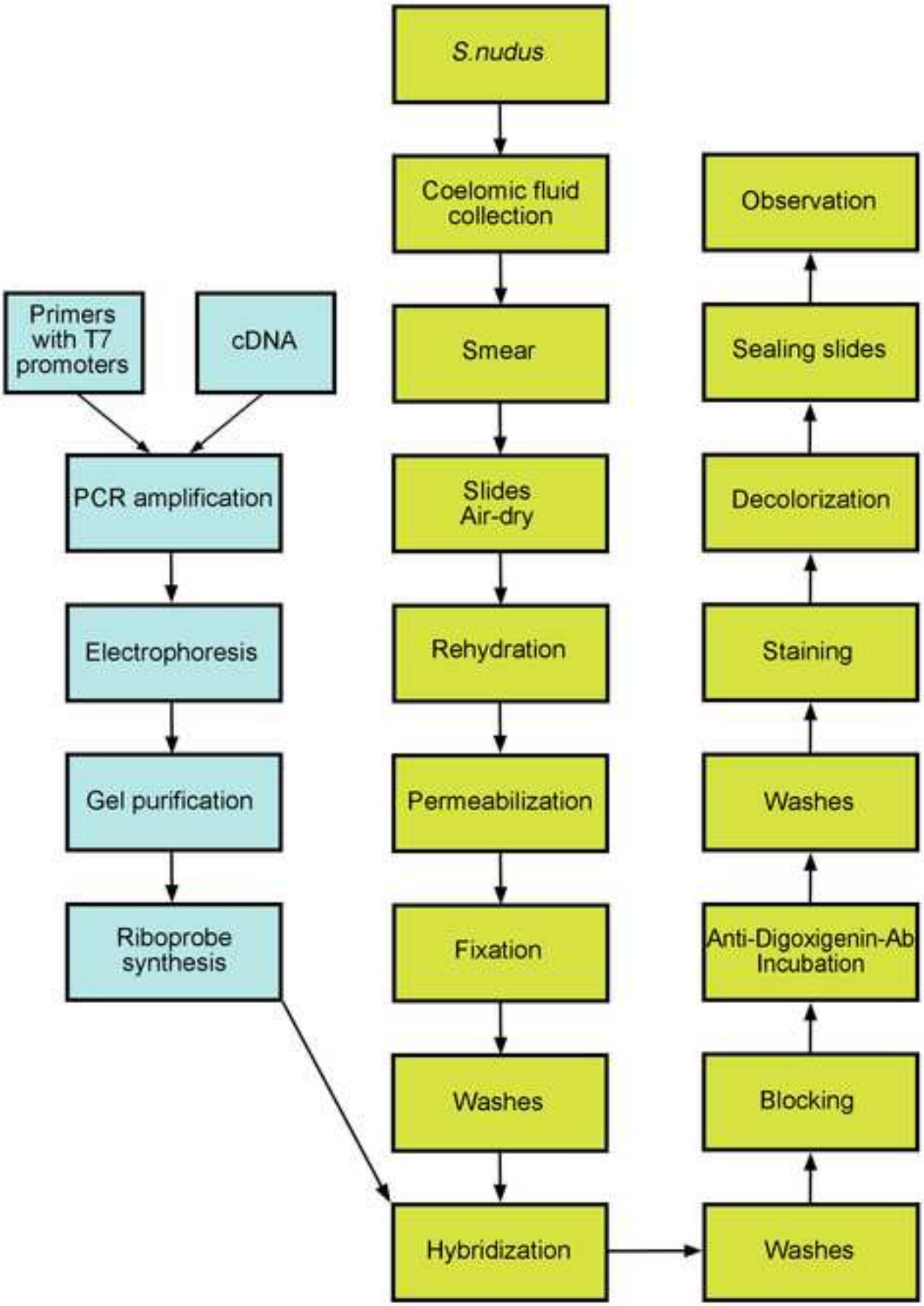

Figure 2

Click here to access/download;Figure;Figure 2.tif

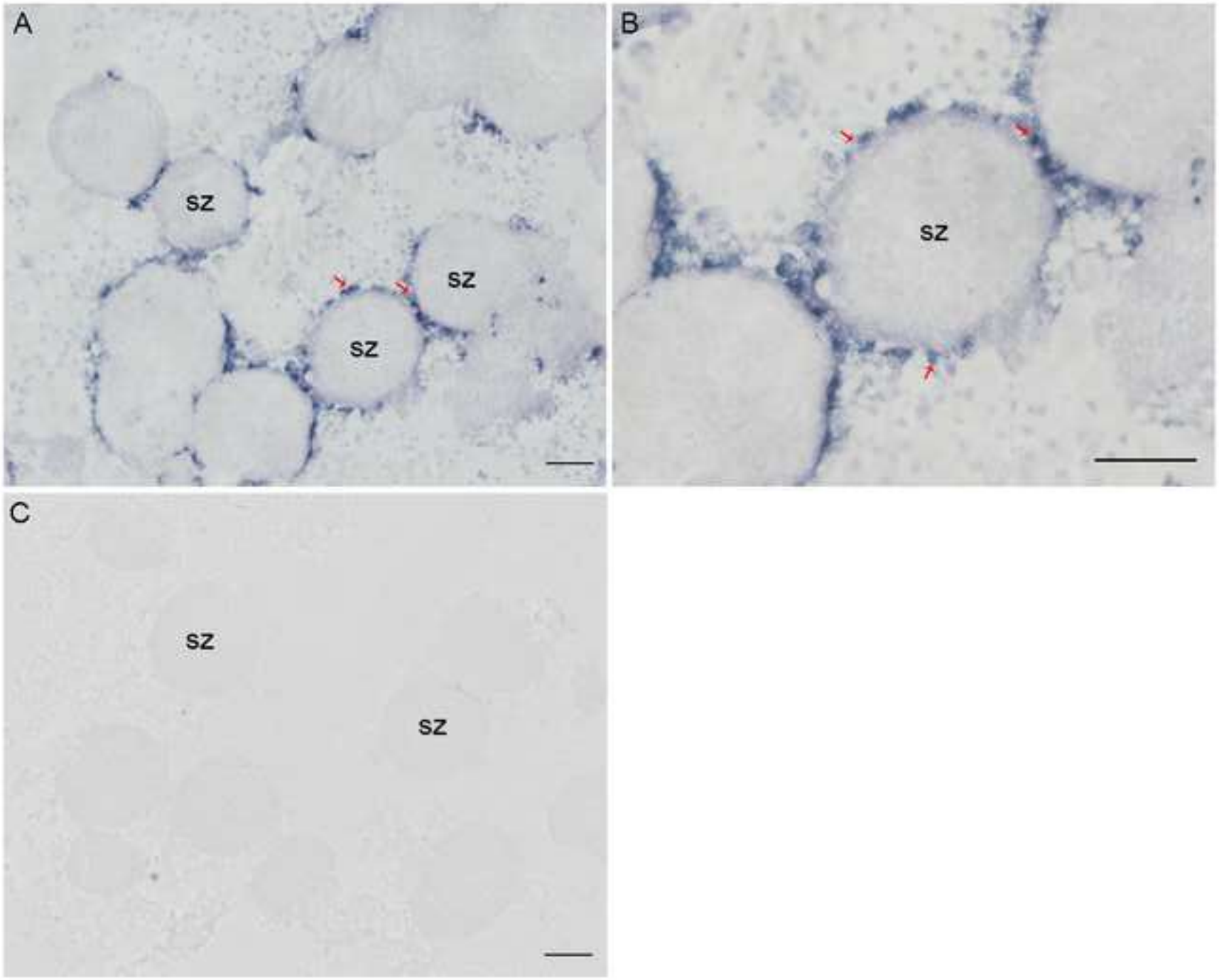

**Table 1 The *dmrt1* primer sequences**

| Name | Sequence (5′—3′) |
|---|---|
| Anti-Probe F | ACAATGTAGCAGGGTTTAATCTTGG |
| Anti-Probe R | TAATACGACTCACTATAGGGAGACTGTTTTCTCTATGCATCCAGTAA |
| Sense-Probe F | TAATACGACTCACTATAGGGAGAACAATGTAGCAGGGTTTAATCTTGG |
| Sense-Probe R | CTGTTTTCTCTATGCATCCAGTAA |

**Table 2 The composition of solutio**

| Reagent |
| --- |
| 5 × TBE (1 L) |
| 10 × PBS (5 L) |
| DEPC-PBS (1 L) |
| 4% PFA in 1 × PBS (100 mL, pH 7.4) |
| 10 mg/mL proteinase K (1 mL) |
| 20 × SSC (1 L) |
| Wet box buffer (50 mL) |
| Hybridization mix (HM, 200 mL) |
| Wash buffer (1 L) |
| MABT (1 L) |
| Blocking buffer |
| Alkaline phosphatase buffer |
| Stop solution |

**ıs used in the ISH protocol**

| Composition |
|---|
| 54 g of Tris, 27.5 g of boric acid and 20 mL 0.5 M EDTA (pH 8.0). |
| 400 g of NaCl, 10 g of KCl, 72 g of $Na_2HPO_4$ and 12 g of $KH_2PO_4$. |
| 1 L of 1 × PBS and 1 mL DEPC. |
| 4 g of PFA and 100 mL PBS. |
| 10 mg of proteinase K. |
| 175.3 g of NaCl and 88.2 g of citric acid trisodium salt. |
| 2.5 mL of 20 × SSC, 22.5 mL of RNase free water and 25 mL of deionized formamide. |
| 100 mL of deionized formamide, 50 mL of 20 × SSC, 10 mg of heparin, 100 mg of tRNA, 0.39 g of citric acid, 1 |
| 50 mL of 20 × SSC, 500 mL of deionized formamide, 450 mL of sterile water and 1 mL Tween-20. |
| 11.6 g of maleic acid, 8.77 g of NaCl, 8.25 g of NaOH and 1 mL Tween-20. |
| 1 × MABT, 2% sheep serum (vol/vol) and 2 mg/mL BSA. |
| 100 mM NaCl, 100 mM Tris HCl (pH 9.5), 50 mM $MgCl_2$, and 0.1% Tween 20 (vol/vol). |
| 0.1 M glycine, pH 2.2. |

200 µL of Tween-20 and RNase free water to 200 mL.

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials.xlsx

| Name of Material/Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Agarose | Biowest | 111860 | |
| Anti-digoxigenin-AP Fab fragments | Roche | 11093274910 | |
| BCIP/NBT alkaline phosphatase color dev | Beyotime | C3206 | |
| Bovine Serum Albumin | Sigma | B2064 | |
| Centrifuge | Eppendorf | 5415R | |
| Citric acid | Sinopharm Chemica | 5949-29-1 | |
| Citric acid trisodium | Sinopharm Chemica | 4/3/6132 | |
| Coverslips | Beyotime | FCGF60 | |
| Deionized formamide | Amresco | 12/7/1975 | |
| Diethyl pyrocarbonate, DEPC | Sigma | D5758 | Noxious substance |
| Digoxigenin (DIG) RNA Labeling Mix | Roche | 11277073910 | |
| DNase I, RNase-free | Invitrogen | 18047019 | |
| EDTA | Sigma | 431788 | |
| Electrophoresis gel imaging | Bio-Rad | Universal Hood III | |
| Ethanol | Sinopharm Chemica | 64-17-5 | |
| Gel extraction kits | Omega | D2500 | |
| Gel-electrophoresis apparatus | Beijing Liuyi Instrun | DYY-6C | |
| Glycerol | Sinopharm Chemica | 56-81-5 | |
| Glycine | Sigma | G5417 | |
| Heparin | Sigma | 8/1/9041 | |
| KCl | Sinopharm Chemica | 7447-40-7 | |
| $KH_2PO_4$ | Sinopharm Chemica | 7778-77-0 | |
| LiCl | Sigma | 203637 | |
| Maleic acid | Sinopharm Chemica | 110-16-7 | |
| Methanol | Sinopharm Chemica | 67-56-1 | Noxious substance |
| $MgCl_2$ | Sinopharm Chemica | 7786-30-3 | |
| $Na_2HPO_4$ | Sinopharm Chemica | 7558-79-4 | |
| NaCl | Biofount | JT0001 | |
| NaOH | Sinopharm Chemica | 1310-73-2 | Corrosive |
| Paraffin film | Bemis Company, In | PM-996 | |
| Paraformaldehyde, PFA | Sigma | 158127 | Noxious substance |

| | | |
|---|---|---|
| PCR Instrument | Life Technology | Proflex |
| Pins | Deli | 16 |
| Pipette | Eppendorf | plus G |
| Poly-D-lysine treated microscope slides | Liusheng | VER_A01 |
| Proteinase K | Sigma | SRE0005 |
| RNase free water | HyClone | SH30538. 02 |
| RNase inhibitor | Roche | 3335399001 |
| *S. nudus* | | |
| Sheep serum | Beyotime | C0265 |
| Slide staining jar | Beyotime | FG010 |
| Slide storage box | Beyotime | FBX011 |
| Small autoclaved scissors | Shuanglu | sku_8330 |
| Spectrophotometers | Thermo Fisher | NanoDrop 2000/2000c |
| T7 RNA polymerases | Roche | 10881767001 |
| Taq DNA polymerase mix (2X) | Thermo Scientific | K1081 |
| Tris HCl | Sigma | RES3098T |
| tRNA | Roche | 10109517001 |
| Tween-20 | Sigma | P1379 |
| Water bath | Zhengzhou Great W | HH-S2 |

**Editorial comments:**

1. Please add 3 seconds of white to the end of the video.

**Response:** Revised accordingly

2. The volume is still too loud. Please examine the volume levels so that they fall between -12 and -6 dB. This may require section-by-section adjustments and not just an overall change.

Response: Revised accordingly. We have edited the volume levels of the relative parts section-by-section.

3. @02:27 There is some noise after the word "agitation" that you may be able to edit out.

**Response:** The noise was removed.

4. @03:00 The words "paraffin film" is incredibly loud and blown out here. You'll have to re-record this line cleanly or reduce the volume in the audio mix.

**Response:** We have re-recorded this line.

5. @05:04 We can hear some papers rustling and the table being bumped after the word, "overnight".

**Response:** The noise was removed.

6. @05:50 Thank you for re-recording this line, but the words "5 minutes per incubation" are also blown out here, similar to "paraffin film" @03:00. This will need to be re-recorded or the volume reduced because as it is now, it is too distorted.

**Response:** We have re-recorded this line.

7. @06:05 Thank you for re-recording this line, but we're hearing the same problem here with "keep the slides in the dark" as above with "paraffin film" and "5 minutes per incubation." For these audio clips, something was not set correctly or not adjusted during recording or assembly into the video. Whatever the cause, these need to be replaced or fixed.

**Response:** We have re-recorded this line.

8. @07:05 "Figure 2A and B)". Same issue. Consider giving this narrator whole lines to say, as well, as it does feel weird to hear part of a line with one voice, and the last few words as another voice, it may work better to share some of the narrator privileges by dividing up whole lines instead of phrases and words.

**Response:** We have re-recorded this sentence.