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Abstract

In situ hybridization is a widely employed technique allowing spatial visualization of gene expression in fixed specimens. It has greatly advanced our understanding of biological processes, including developmental regulation. In situ protocols are today routinely followed in numerous laboratories, and although details might change, they all include a hybridization step, where specific antisense RNA or DNA probes anneal to the target nucleic acid sequence. This step is generally carried out at high temperatures and in a denaturing solution, called hybridization buffer, commonly containing 50% (v/v) formamide – a hazardous chemical. When applied to the soft-bodied hydrozoan medusa *Clytia hemisphaerica,* we found that this traditional hybridization approach was not fully satisfactory, causing extensive deterioration of morphology and tissue texture which compromised our observation and interpretation of results. We thus tested alternative solutions for in situ detection of gene expression and, inspired by optimized protocols for Northern and Southern blot analysis, we substituted the 50% formamide with an equal volume of 8 M urea solution in the hybridization buffer. Our new protocol not only yielded better morphologies and tissue consistency, but also notably improved the resolution of the signal, allowing more precise localization of gene expression and reducing aspecific staining associated with problematic areas. Given the improved results and reduced manipulation risks, we tested the urea protocol on other metazoans, two brachiopod species (*Novocrania anomala* and *Terebratalia transversa*) and the priapulid worm *Priapulus caudatus,* obtaining a similar reduction of aspecific probe binding. Overall, substitution of formamide by urea during in situ hybridization offers a safer alternative, potentially of widespread use in research, medical and teaching contexts. We encourage other workers to test this approach on their study organisms, and hope that they will also obtain better sample preservation, more precise expression patterns and fewer problems due to aspecific staining, as we report here for *Clytia* medusae and *Novocrania* and *Terebratalia* developing larvae.

Keywords

In situ hybridization; urea; formamide; *Clytia*; *Novocrania; Terebratalia; Priapulus*

Introduction

In Situ Hybridization (ISH) is a widely employed and powerful technique, allowing localization of specific nucleotide sequences in DNA or RNA strands within cells or tissues. The coupling of genetic and histological information provides a highly informative view of spatial gene expression. Nucleic acids have the fundamental property of pairing to complementary sequences, which in the case of ISH probes are exogenously synthesized and labeled through the incorporation of chemically modified nucleotides, allowing for the detection of known target sequences in the endogenous gene or mRNA. The technique was developed in the 60s (Pardue and Gall, 1969), and has since proven invaluable in cell and developmental biology research, as well as in medical diagnostics.

ISH has been successfully applied to animals, plants and bacteria, and over the years numerous protocols have been developed, tailored to specific needs, such as the detection of non-coding RNA, or for different sample types including whole embryos, tissue sections, or cell preparations. A number of alternative approaches can be used for labeling and subsequent detection of probes. Historical use of probes incorporating radioactive nucleotides has today been largely superseded by safer alternatives, involving nucleotides linked to biotin or Digoxigenin (DIG) (Tautz and Pfeifle, 1989), or to fluorescent tags.

The hybridization step, central to the process, is carried out at high temperatures, promoting the breaking of hydrogen bonds and destabilizing the nucleic acid strands. Usually a temperature in the 55° to 65°C range is chosen, aiming at a compromise between sensitivity and specificity of the probe-target annealing reaction. Temperatures as high as 72°C can be used (Blackshaw, 2013). The ideal temperatures for denaturation and annealing depend on the nature of the target nucleic acid strands: denaturation or melting temperatures (Tm) are determined by the base composition of the target sequence (C-G Watson-Crick bonds are more stable than A-T), while ideal hybridization temperatures fall about 25°C below Tm (Marmur and Doty, 1961). Unfortunately, the long incubations usually performed to allow probe penetration into the specimen increase the risk of nucleic acid degradation and tissue damage in the samples, and much effort has been therefore devoted to finding methods to lower hybridization temperatures. Early reports modified parameters such as salt concentration, pH, and solvents to modulate the efficiency and stringency of the hybridization process, and to lower the reaction temperature by destabilizing the structured organization of DNA or RNA molecules. Various organic solvents found to effectively destabilize nucleic acid structure included guanidinium chloride, salicylate, formamide, dimethyl sulfoxide (DMSO), N,N'-dimethylformamide (DMF), a variety of alcohols (for

example see Rice and Doty, 1957; Marmur and Ts'o, 1961; Hamaguchi and Geiduschek, 1962; Herskovits, 1962; Levine et al., 1963), urea and several of its derivatives, or sodium hydroxide, used in the first hybridization in situ experiments on *Xenopus* oocytes (Pardue and Gall, 1969).

The first protocols for ISH indeed achieved nucleic acid denaturation either with high temperatures or with NaOH or salts (John et al., 1969; Buongiorno-Nardelli and Amaldi, 1970; Barsacchi and Gall, 1972; Gall, 2016). Only few years later, researchers favored instead the addition of 50-70% formamide in the hybridization buffer, recently found to lower hybridization temperature and efficiently denature DNA/RNA (see for example (Barbera et al., 1979; Bauman et al., 1980; Gerhard et al., 1981; Hafen et al., 1983; Levine et al., 1983; Braissant and Wahli, 1998; Brown, 1998). This organic solvent was considered particularly useful, for its ability of denaturing and renaturing DNA at room temperature (Hutton, 1977; Marmur and Ts'o, 1961; McConaughy et al., 1969), a property that allowed the generation of the first DNA-RNA hybrids (Bonner et al., 1967).

Formamide is today standardly employed in different hybridization methods, either in situ hybridization or Northern and Southern blotting, and generally provides reliable results. However, it is also a very hazardous chemical, causing both short term effects such as respiratory tract irritation, headache and nausea, and long term damage to internal organs and to reproduction ((Fail et al., 1998; George et al., 2002, 2000; Gleich, 1974; Stula and Krauss, 1977; Merkle and Zeller, 1980; Kennedy and Short, 1986), see also Table 1 for further details). It is rapidly absorbed orally, via inhalation or skin contact, and since in experimental animals formamide has been shown to have embryotoxic and teratogenic effects (Merkle and Zeller, 1980; George et al., 2000, 2002), pregnant women are considered to be particularly at risk (*European Chemical Agency. Proposal for identification of a substance as a CMR CAT 1A or 1B, PBT, vPvB or a substance of an equivalent level of concern. Formamide*). Moreover, the generally high reaction temperatures used for ISH pose an additional threat, since augmented evaporation increases the risk of inhalation. These hazards mean that handling of samples and of waste has to be carefully controlled and managed (*CICAD 31: N,N-Dimethylformamide*, 2001).

The ISH technique is a well-established method for detecting gene expression in the hydrozoan *Clytia hemisphaerica*, and has proven extremely useful for understanding embryonic development, oogenesis, and the biology of adult structures such as the tentacle bulb (Chevalier et al., 2006; Denker et al., 2008; Leclère et al., 2012; Lapébie et al., 2014; Kraus et al., 2015). Unfortunately, the prolonged, high-temperature, hybridization step is rather aggressive for the medusa form, particularly for the fragile umbrella, rich in extracellular matrix. While other

morphological features of the medusa, such as the feeding manubrium, the gonads and the tentacle bulbs (see Fig. 1A and 1B), retain their overall structural integrity, the umbrella becomes deformed and shrunken (Fig. 1C), thus impairing the study of finer elements, such as the nervous system network underlying the umbrellar epithelia. This limitation, which different fixation methods could not overcome, prompted us to question the standard hybridization step. We searched for alternative hybridization buffer composition that could improve sample preservation and, we set to target formamide as the most abundant and aggressive reagent.

During the early years of research on nucleid acids, urea was identified as an efficient organic solvent (Herskovits, 1963), still mainly employed as a denaturing agent in PAGE (Polyacrylamide Gel Electrophoresis) methods (Summer et al., 2009). Urea and formamide share similar properties, and have been long successfully employed as equivalents in a number of techniques (Kourilsky et al., 1971), including Fluorescent In Situ Hybridization (FISH) on bacteria (for a recent report see (Fontenete et al., 2016)), protein denaturation (e.g. (Lim et al., 2009)), or as clearing agents for tissue imaging (e.g. ScaleS and ClearT methods respectively, reviewed in (Azaripour et al., 2016)).

Here, we present a formamide-free in situ hybridization protocol for the hydrozoan *Clytia hemisphaerica*, in which the use of urea as a denaturing agent not only improves the overall morphology of specimens, but can also improve the sensitivity of the detection. This substitution allows for a safer and easier procedure too, with reduced risks both for the operator and the environment. In addition, by successfully assessing gene expression in two developing brachiopods, *Novocrania anomala* and *Terebratalia transversa*, and in embryos of the priapulid worm *Priapulus caudatus,* we show that this alternative urea-containing hybridization buffer can represent a general useful option for in situ hybridizations in other metazoan species.

Methods

Animal culture/collection

The *Clytia hemisphaerica* (Linnaeus, 1767) Z4B strain used in this study is cultured in artificial sea water, under controlled conditions of temperature (20°C), pH and water flow, in our in-house aquarium system (Houliston et al., 2010). Medusae were fed with newly hatched *Artemia* and grown until fully mature (2-3 weeks from release from gonozooid polyp) before fixation.

Priapulus caudatus (Lamarck, 1816) collection was performed as described in (Martín-Durán and Hejnol, 2015), while collection of *Terebratalia transversa* (Sowerby, 1846) and *Novocrania*

anomala (O. F. Müller, 1776) was done as in (Santagata et al., 2012) and (Martín-Durán et al., 2016), respectively.

Clytia hemisphaerica in situ hybridization protocol

Protocols were adapted from (Lapébie et al., 2014) and from Takeda et al. (https://doi.org/10.1101/140160) for chromogenic (CISH) and fluorescent in situ hybridization (FISH), respectively.

Medusae were relaxed and fixed on ice with a pre-chilled solution of 3.7% formaldehyde plus 0.4% glutaraldehyde in 1X PBS (Phosphate-Buffered Saline), for two hours (CISH fixation) or fixed for 36 hours at 18°C with 3.7% formaldehyde in HEM buffer (0.1M HEPES pH 6.9, 50mM EGTA pH 7.20, 10mM $MgSO_4$). Specimens were washed thoroughly with 1X PBST (1X PBS plus 0.1% Tween-20), and stepwise dehydrated to 100% methanol, and finally stored at -20°C. Samples were re-hydrated for 15 minutes with 50% methanol/ PBST, followed by three PBST washes.

The traditional hybridization solution that we used for comparison contained a 50% volume of formamide, plus other reagents commonly employed in hybridization mixes (final proportions are provided; further details about reagents are included in the Supplementary Material #2): 5X SSC (Saline Sodium Citrate, a buffer solution at pH 7.00); 1% dextran powder (which acts as a volume-excluding polymer to concentrate the probe, thus promoting hybridization rate); 50 µg/ml of tRNA (a blocking agent, reducing non-specific binding); 50 µg/ml of heparin (which reduces background staining; Singh and Jones, 1984); 1% SDS (Sodium Dodecyl Sulfate, a detergent permeabilizing membranes; see Shain and Zuber, 1996), and milliQ purified H_2O to volume (Fig. 1C, 2A, 2C, 2E, 2G, 2I, 2K, 2M, 2O, 2P, 2R, 2T). In our new protocol, the formamide was substituted with an equivalent volume of freshly prepared urea solution (8M urea, dissolved in milliQ H_2O), so that the final hybridization mix contained 4M urea, plus the same reagents listed above (5X SSC; 1% dextran; 50 µg/ml tRNA; 50 µg/ml heparin; 1% SDS), see Figures 1D, 2B, 2D, 2F, 2H, 2J, 2L, 2N, 2Q, 2S and 2U. The concentration of urea was determined on the basis of available literature. In particular, Simard et al. (2001) demonstrated that for Northern blot a 2-4M urea-containing solution provided the best signal, while higher concentrations significantly decreased the sensitivity of the hybridization. Similar values were found by Søe and colleagues (2011), who showed that a 4M urea-containing hybridization buffer provided the best detection of low-copy miRNAs in mouse brains. Samples were gradually transferred to the hybridization solution at room temperature. First they were incubated for 10 minutes in a 50% hybridization buffer/ PBST solution, then for 20 minutes with hybridization buffer alone, and then pre-

hybridized at 58°C for two hours. Probes (details about synthesis are provided in Supplementary Materials #1) were added to a final concentration of 0.1-1 ng/µl, and hybridized at 58°C for 72 hours (previous experiments demonstrated that 48 hour incubations can produce satisfactory results, in particular for strongly expressed genes). Samples were then transferred to progressively stringent washes, still at 58°C, as follows: three washes of 30 minutes with (4M urea, 0.1% Tween-20, 5X SSC, milliQ H_2O), likewise with (2M urea, 0.1% Tween-20, 2X SSC, milliQ H_2O), and finally twice for 30 minutes with (0.1% Tween-20, 2X SSC, milliQ H_2O).

For CISH detection, samples were transferred to MABT (Maleic Acid Buffer, containing Tween-20), and washed twice with MABT (20 minutes), pre-treated with blocking solution (MABT/ 1% Blocking Reagent (Roche)) for 1 hour, and finally incubated with the appropriate antibody (anti-DIG-AP, 1:2000 in blocking solution) overnight at 4°C. Samples were then thoroughly washed with MABT (six washes of 15 minutes) and transferred to NTMT buffer (NaCl, Tris-HCl at pH 9.5, $MgCl_2$, Tween-20). The signal was detected with a colorimetric NBT/BCIP reaction (0.08 mg/ml of Nitro Blue Tetrazolium and 0.1 mg/ml of 5-Bromo-4-Chloro-3-Indolyl-Phosphate), which was then stopped with a rapid milliQ H_20 rinse, followed by a 1X PBS wash. Samples were post-fixed with 3.7% formaldehyde in 1X PBS for 30 minutes, rinsed with 1X PBS, and finally transferred to glycerol for imaging and long-term storage.

For FISH detection, samples were transferred to MABT, and incubated overnight with the appropriate antibody, peroxidase conjugated (anti-DIG-POD, 1:2000 in blocking solution). Samples were then washed twice in fresh color reaction buffer (0.0015% H_20_2 in PBS) for 30 minutes. Signal was developed with fluorophore-conjugated tyramide kit (1:400 in color reaction buffer) for one hour. Samples were washed with 1X PBS, stained with Hoechst 33528 (from Sigma, used at 0.3 µg/ml in 1X PBS) for 30 minutes, rinsed with 1X PBS, and finally transferred to Citifluor AF1 (Electron Microscopy Sciences) for storage and imaging. The complete protocol is provided in the Supplementary Material #2.

Novocrania, Terebratalia **and** ***Priapulus*** **in situ hybridization protocols**

The protocol was adapted from (Hejnol, 2008), with the following modifications: proteinase K digestion (before hybridization) was followed by a post-fixation with 3.7% formaldehyde and 0.2% glutaraldehyde in PBST, the hybridization solution contained 1% dextran, and, finally, the formamide in the hybridization buffer and stringent-wash buffer, was replaced by 8M urea (with a final concentration of 4M urea). The complete protocol is provided in the Supplementary Material #3.

Image acquisition and processing

Full-sized medusae images were taken on Leica M205 FA and M165 FC stereomicroscopes. Colorimetric images were taken on a Zeiss Axio Imager A2. All fluorescent images were taken on a SP8 Leica confocal microscope, using the same image acquisition and laser parameters. The composite images of medusae (Fig. 1C and 1D) were assembled with Photoshop CS5, as follows: images were converted to black-and-white, transformed into outlines with the Stylize filter, opacity was reduced to 20%, and the resulting images were superimposed, generating global shapes. Colorimetric ISH images were adjusted with Photoshop CS5, while fluorescent images were processed with the same noise reduction parameters using the default settings of the proprietary Leica software (LasX). *Novocrania, Terebratalia and Priapulus* embryos were imaged with Axiocam, on an Axioscope 2, and processed with the Axiovision software.

Results

Improved medusa morphology following in situ hybridization

The medusa stage of the hydrozoan *Clytia hemisphaerica* (Fig. 1A) displays tetraradial symmetry, with four radial canals running from the oral manubrium to the ring of tentacle bulbs at the bell margin, and four gonads positioned on the radial canals. The transparent umbrella is composed of an exumbrellar layer and a subumbrella, separated by a thick layer of acellular mesoglea. The central manubrium, leading to the digestive pouch, is rather short and is organized according to the general tetraradial symmetry. A circular canal follows the periphery of the bell, connecting the four radial canals and the tentacle bulbs, numbering 16 in the adult animal. A thin velum, a bi-layered epithelium typical of hydrozoan medusae, extends from the bell margin and contributes to swimming (Leclère et al., 2016). The aldehyde-based fixation process used routinely for ISH reliably preserved the shape and the size of the live animal, which at the adult stage measures about 1 cm in diameter (Fig. 1B). However, the standard, formamide-based, in situ hybridization treatment invariably caused an extensive shrinking of the umbrella, with a marked alteration of body proportions. The umbrella appeared folded and heavily shrunken, while the more conspicuous elements, such as the manubrium, the gonads and the tentacle bulbs did not appear to be significantly affected, allowing the overall organization of the medusa to be maintained (Fig. 1C). The composite image in Fig. 1C, generated by the superposition of 50 animals, highlights their irregular morphology, and the

damage to the umbrella. This deformation, along with a general stiffening of tissues occurring during the processing, impaired the analysis of gene expression and the recognition of fine features. In contrast, the urea-based treatment preserved a better medusa morphology, as shown by the superposition of 50 different jellyfish (Fig. 1D), although the shrinking could not be completely avoided. The improved preservation of tissues, coupled with a more flexible consistency of medusae following the urea-based protocol, greatly facilitated the observation of our specimens.

A more sensitive technique for gene expression analyses

Nervous system-related genes provide a reliable way to assess the precision of mRNA detection, given their cell type-specific expression. We thus chose to compare the expression patterns for *mcol3/4a* (*minicollagen 3/4a* in Denker et al., 2008), *RFamide* (also called *pp5)*, and *drgx* and *six3/6* (Kraus et al., 2015). In all cases, formamide and urea variants gave comparable expression patterns, demonstrating that 4M urea could efficiently substitute for the 50% formamide during hybridization steps (results are shown in Figure 2). The urea-based protocol produced sharper staining patterns compared to the formamide one, particularly in the case of isolated cells, as for example showed by *mcol3/4a* expression in nematoblasts (the cells that will produce the nematocysts) at the base of the manubrium, and in the tentacle bulbs (compare Fig. 2A and 2B, 2E and 2F), where single positive cells could be more easily distinguished within the tissues.

For the genes tested, the urea method proved to be overall more sensitive than the traditional protocol. Testing a 10-fold reduced probe concentration (0.05 ng/µl), the *mcol3/4a* signal could no longer be detected in the manubrium using the formamide method (Fig. 2C), while clear staining could still be obtained using urea (Fig. 2D). We cannot currently assess whether the poorer staining was due to probe degradation, which could for example occur if the deionized formamide had lost purity (in our experiments, recently opened bottles of deionized formamide were used, kept at 4°C) but not in the urea- based hybridization solution.

The greater sensitivity of the urea method was particularly relevant for *RFamide* expression, where, using the same probe and color development conditions, we could reveal an unexpected complexity of the neural network in the manubrium, in the circular canal, and in the subumbrella (manubrium shown in Fig. 2I and 2J, circular canal in Fig. 2K and 2L).

An increased signal-to-noise ratio

Side-by-side comparison of staining patterns produced by formamide or urea protocols highlighted the tendency of the formamide-based hybridization method to generate aspecific staining of some medusa structures. In the case of fluorescent in situ hybridization, which is a highly sensitive method, the aspecific staining generated by the formamide protocol was in some cases so strong as to mask the true signal (for instance compare staining of *RFamide* neurons in the subumbrellar and radial canal regions in Fig. 2P and 2Q). Extensive previous experience with the colorimetric method had revealed a range of reproducible non-specific signals, including diffuse staining of the tentacle bulbs (exemplified by *mcol3/4a* detection in Fig. 2E, 2G), and superficial, punctate staining of the margin of the velum (see for example the *drgx* detection in Fig. 2M and *six3/6* in Fig. 2O), and on the surface of gonads (as illustrated by *drgx* detection in Fig. 2T). The non-specific nature of the signal was easily recognizable for its rapid appearance and its superficial localization over epithelia, and further confirmed using control sense-strand probes (see the control for *mcol3/4a* in Fig. 2G, which shows a strong signal in the tentacle bulb and in the endoderm of tentacle). These strong signals forced premature arrest of the detection reaction, with the risk of under-developing the 'true' expression patterns. These issues were resolved using the urea method, as demonstrated by the sharp signal obtained at the level of the tentacle bulb with the *mcol3/4a* probe, where the endoderm of the tentacle appears clear of background (Fig. 2F), and the absence of spots on the surface of velum and gonad (Fig. 2N and 2U, respectively). Moreover, control ISH performed in parallel with a sense probe (for *mcol3/4a*, Fig. 2H) produced a clean result, devoid of any non-specific staining.

To summarize, the urea ISH protocol for *Clytia* medusae not only allowed us to identify more confidently sites of gene expression, but also to continue the color development reaction until details of the expression patterns had been completely revealed.

The urea method is a reliable alternative for multiple species

The modified, urea-based, ISH protocol detailed in this study is safe and simple to implement, and we wondered whether it could be generally applicable across metazoan species.

We thus tested it on different animals and on different developmental stages, posing various experimental challenges. We included two Brachiopoda species *(Novocrania anomala* and *Terebratalia transversa*) and a priapulid worm (*Priapulus caudatus*), for which in situ hybridization protocols had been successfully established, but where gene expression analysis were at times hindered by aspecific staining. Similarly to *Clytia*, we used the already published

nervous system-related genes, *nk2.1* and *otx (orthodenticle*), as a reference (Martín-Durán et al., 2016, 2012; Martín-Durán and Hejnol, 2015).

In all three species, the urea-based hybridization buffer produced a specific signal, compatible with the results already described using the formamide-based hybridization buffer particularly in *P. caudatus,* where the resolution appeared substantially equivalent. Examples included expression of *nk2.1* in the oral ectoderm territory (formamide and urea-protocol shown in Fig. 3M and 3N, respectively), and of *otx* in the oral and ventral side (Fig. 3O and 3P, formamide and urea respectively). A decisive difference was observed for older stages of the two brachiopod larvae, where the central part of the body includes a typical site of aspecific staining, associated with the shell-secreting gland - shown by the formamide in situ hybridization images for *nk2.1* (Fig. 3A and 3G) and *otx* (Fig. 3C, 3E, 3I, 3K). This strong staining was never observed for specimen processed using the urea protocol (compare Fig. 3B, 3D, 3H, 3J). The aspecific nature of the glandular staining was confirmed by the signal seen in the corresponding sense-probe formamide control (see Fig. 3E and 3K), which was markedly absent in the urea-control (Fig. 3F and 3L). A possible explanation for this difference is that the shell-gland staining results from probe trapping, a common artifact, and that urea buffers could improve the washing and reduce the retention of reagents due to greater tissue permeabilization.

These results demonstrate not only that our urea protocol for in situ hybridization can be successfully applied to other species, but importantly that it can prove very useful in reducing different types of non-specific signal associated with particular structures, as exemplified by the adult medusa and the late brachiopod larvae.

Discussion

The urea-based in situ hybridization protocol described in this study represents a powerful alternative to the standard formamide-based in situ hybridization techniques. In both the hydrozoan medusa *Clytia hemisphaerica* and two brachiopod species, *Novocrania anomala* and *Terebratalia transversa*, the urea-based hybridization buffer effectively boosted signal detection, reduced aspecific staining and improved specimen morphology, allowing for a more confident interpretation of results.

The common in situ hybridization techniques routinely employed to assess gene expression in numerous metazoan models, such as *Xenopus*, *Danio*, *Nematostella* or *Mus*, typically employ a

hybridization buffer composed of a 50% volume of formamide. Formamide is a dangerous chemical, especially at high temperatures, which increase evaporation and the risk of exposure to hazardous vapors. Extreme care is therefore needed during manipulation and for waste disposal, which can be particularly problematic in some field work or teaching contexts. For these safety and environmental reasons several reports have recently questioned the extensive use of formamide, asking if a less toxic option could be found (see Table 1 for a safety comparison of formamide and urea, and Table 2 for a survey of available literature). These studies were mostly aimed at medically-oriented research, for example for developing *in vivo* pathogen diagnostic techniques.

For ISH, formamide is widely used as a denaturing agent, but in other types of gene expression analysis, such as Northern, Southern or Western blots, urea is often employed for denaturing both proteins and nucleic acids. Indeed, the denaturing properties of urea have been known and investigated since the 1960s, and the efficiency of urea and formamide in hybridizations to RNA probes has already thoroughly been tested for blotting applications (Simard et al., 2001). It was demonstrated not only that urea could effectively replace formamide in detecting RNA, but also that the best results were obtained when urea concentrations were comprised between 2M and 4M. Above this value the sensitivity of detection was markedly reduced, probably due to the high viscosity of the solution (Hutton, 1977). Our observations in the laboratory paralleled this finding, with the urea-based hybridization solution being more viscous than the formamide one. This difference might be relevant while adapting the protocol to new organisms.

Formamide and urea share similar denaturing properties and are broadly employed for numerous applications. Nevertheless, their mechanisms of action are still incompletely understood, probably due to the multiple factors affecting the efficiency of the reaction. Formamide lowers the melting temperature of DNAs by 2.4- 2.9°C (per mole of formamide) - with an efficiency depending on the properties of the target nucleic acid strands, such as their G+C content, the helix topology and the state of hydration (Blake and Delcourt, 1996). This solvent weakens hydrogen bonds, ultimately allowing for lower hybridization temperatures, maintaining similar high stringencies (Casey and Davidson, 1977; Sadhu et al., 1984; Robertson and Vora, 2012). Generally, the more concentrated the formamide, the higher is the stringency of reaction, but it was shown that, similarly to urea, an excess of solvent causes a dramatic drop in probe binding and signal detection (Manz et al., 1992; Bond and Banfield, 2001). Non-specific signal is a common artifact, and stringency can be further improved through several post-hybridization washes, which remove the excess probe and disrupt any incorrectly paired

duplexes, for example weak base pairs between Guanine and Uracil in RNA (Uhlenbeck et al., 1971; Lomant and Fresco, 1975). The stringency of the washing buffer can be further controlled by lowering the concentration of salt, instead of using formamide, thus reducing the volume of toxic waste (Lathe, 1985).

Another factor affecting the signal-to-noise ratio in ISH is the purity of the formamide in the hybridization buffer, and numerous protocols recommend using deionized formamide. The reason for choosing a deionized solvent is that formamide solutions become acidic with time, due to the hydrolytic breakdown of formamide to formic acid and ammonium formate, responsible for attacking the phosphodiester bonds of RNA strands and degrading the larger RNA molecules in particular (Chow and Broker, 1989). The purification step removes those breakdown products, which will then take time to reform (this is why, for sensitive applications, it is generally recommended to use freshly opened bottles or to deionize the formamide from older ones). Our, previous experience did not show any difference between deionized or non-deionized formamide, and in the *Clytia* laboratory we routinely use recently opened bottles of deionized formamide, kept at 4°C.

Due to the widespread use of formamide, fewer studies have addressed the mechanism of action of urea. Urea can substantially lower the melting temperature of DNA, with values approaching 2°C reduction per mole of urea (Hutton, 1977), and thus slightly lower than the decrease that can be obtained with formamide. Recently it was shown that, as hypothesized previously, urea can interact with water and with both the polar and nonpolar components of nucleotides. It forms multiple hydrogen bonds with the RNA bases, and generates stacking interactions with them, ultimately disrupting the base-pair interactions and causing a destabilization of the structure of the RNA molecules (Herskovits and Bowen, 1974; Priyakumar et al., 2009; Lambert and Draper, 2012).

In our test species, the sensitivity of detection increased when formamide was replaced by urea, in line with a similar result previously observed for a FISH protocol developed for detecting *Helicobacter pylori* in gastric biopsies (Fontenete et al., 2013). This might be due to an additional permeabilization action of urea (Lim et al., 2009; Huang et al., 2011), which could enhance probe penetration in the tissues. An enhanced permeabilizing activity for urea could explain the improved detection we obtained in urea-treated medusae, for example demonstrated using low *mcol3/4a* probe concentrations (see Fig. 2C and 2D).

The urea alternative appears to be a reliable option for routine in situ hybridization, and has already been successfully tested in multiple species, including *C. hemisphaerica*, *N. anomala*, *T.*

transversa, *P. caudatus* (this study), and also the scyphozoan jellyfish *Aurelia aurita* (M. Manuel and T. Condamine, personal communication), the acoel *Hofstenia miamia* (L. Ricci, personal communication), and mouse oocytes (M.H. Verlhac and M. Manil-Segalen, personal communication). Despite broad reproducibility, we would recommend performing an initial comparison between the two protocols, in order to verify the reproducibility of the gene expression patterns detected.

Overall, substitution of formamide by urea in situ hybridization offers a safer alternative protocol, potentially useful in a broad spectrum of research, medical and teaching contexts. We encourage other workers to test this approach on their study organisms, and hope that they will also obtain more informative and sharp expression patterns, as we saw with *Clytia hemisphaerica*, *Novocrania anomala* and *Terebratalia transversa.*

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Figures

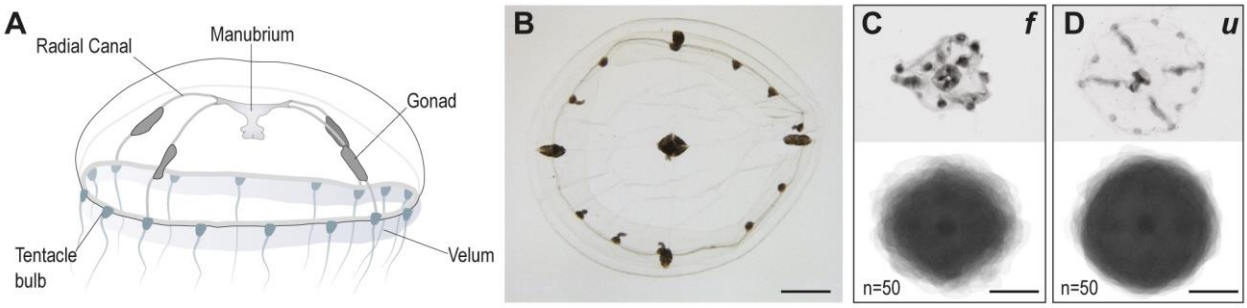

Figure 1. The morphology of *Clytia* medusae is better preserved after urea-based in situ hybridization (u), with respect to the classical, formamide-based, protocol (f).

(A) Anatomy of *Clytia* medusa: the tetraradial symmetry is evident in the four radial canals emerging from the manubrium (stomach and mouth part), crossing the umbrella, from which the four gonads develop. An acellular, thick, mesoglea separates exumbrella and subumbrella layers. (B) Oral view of a medusa, post-fixation. The fixation process preserves morphology and size of the living animal. (C, D) Sample individual pictures (top) and composite images generated by the superposition of 50 images (bottom) of medusae following formamide-based (C) or urea-based (D) hybridization steps. Scale bars, 1 mm.

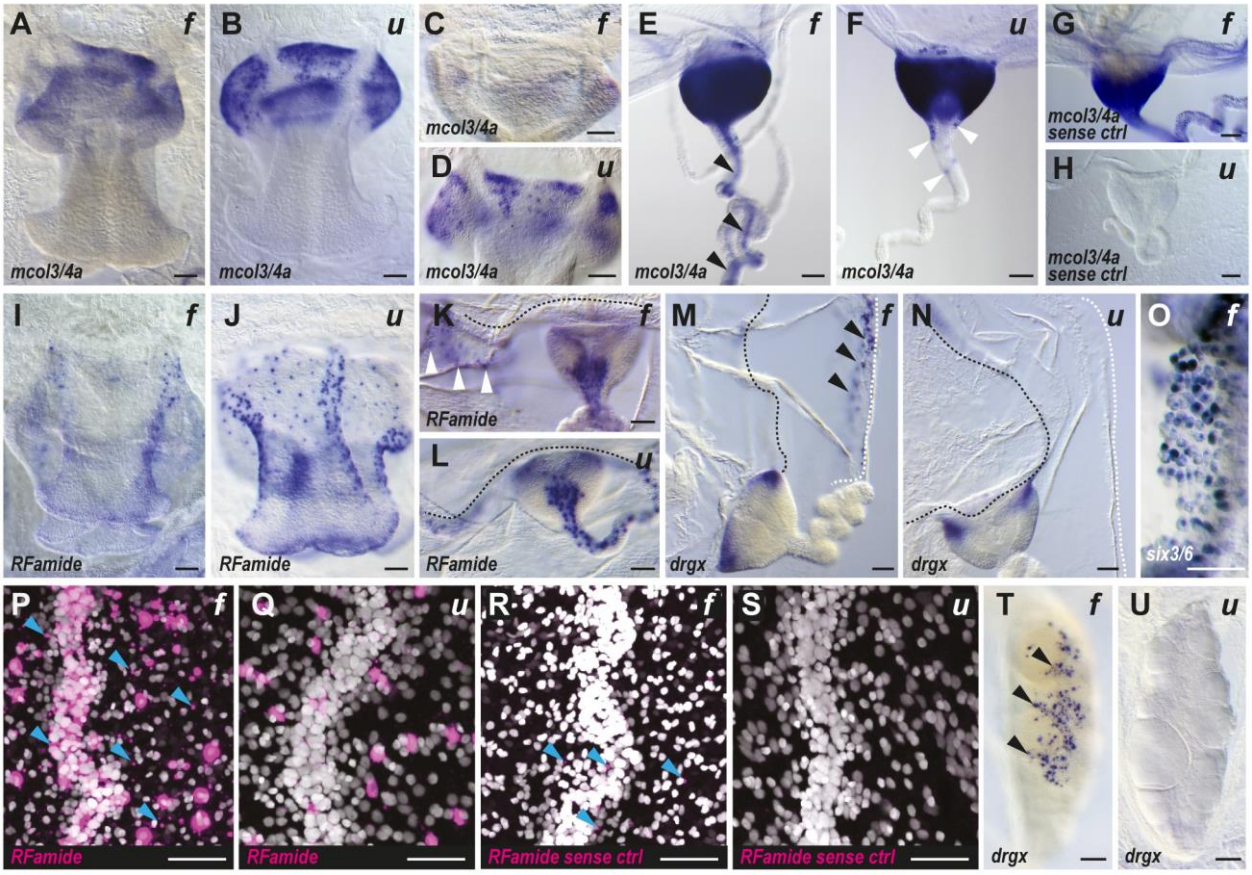

Figure 2. Improved sensitivity and signal-to-noise ratio with the urea-based in situ hybridization method on *Clytia* medusa.

(A-O,T,U) Colorimetric and (P-S) fluorescent (signal in magenta, nuclear Hoechst staining in white) in situ hybridization results, comparing formamide-based (f) and urea-based (u) methods. Antisense (A-F, I-Q, T-U) and sense (G, H, R, S) probe gene names are indicated at the bottom left of each picture. The pictures show: (A, B, I, J) whole manubrium with oral opening at the bottom; (C, D) aboral, basal, side of the manubrium; (E-H, K, N) tentacle, tentacle bulbs and portions of the bell margin (black dotted line in L-N) and velum (white dotted line in M and N); (P-S) portions of radial canals running from top to bottom and nearby subumbrella; (O) high magnification of velum margin; (T, U) female gonad with oocytes. Black arrows point to aspecific staining seen after formamide-based ISH in: the tentacle endoderm (in E), velum epithelium (in M) and gonad ectoderm (T). White arrows highlight specific single cell staining produced with the urea-based method in the tentacle ectoderm (in F) and radial canal (in K). Blue arrows indicate few examples of aspecific fluorescent staining (in P and R). In (C and D) the concentration was decreased by 10 fold compared to (A and B), notice how the specific *mcol3/4a* signal at the base of the manubrium is lost with the formamide method (C), while it is still detectable with the urea

method (D). The aspecific staining on tentacle using *mcol3/4a* sense control probe, as shown in (G), was obtained in many but not all analyzed medusae. (P-R) the four fluorescent images were taken with the same settings. Scale bars, 50 µm

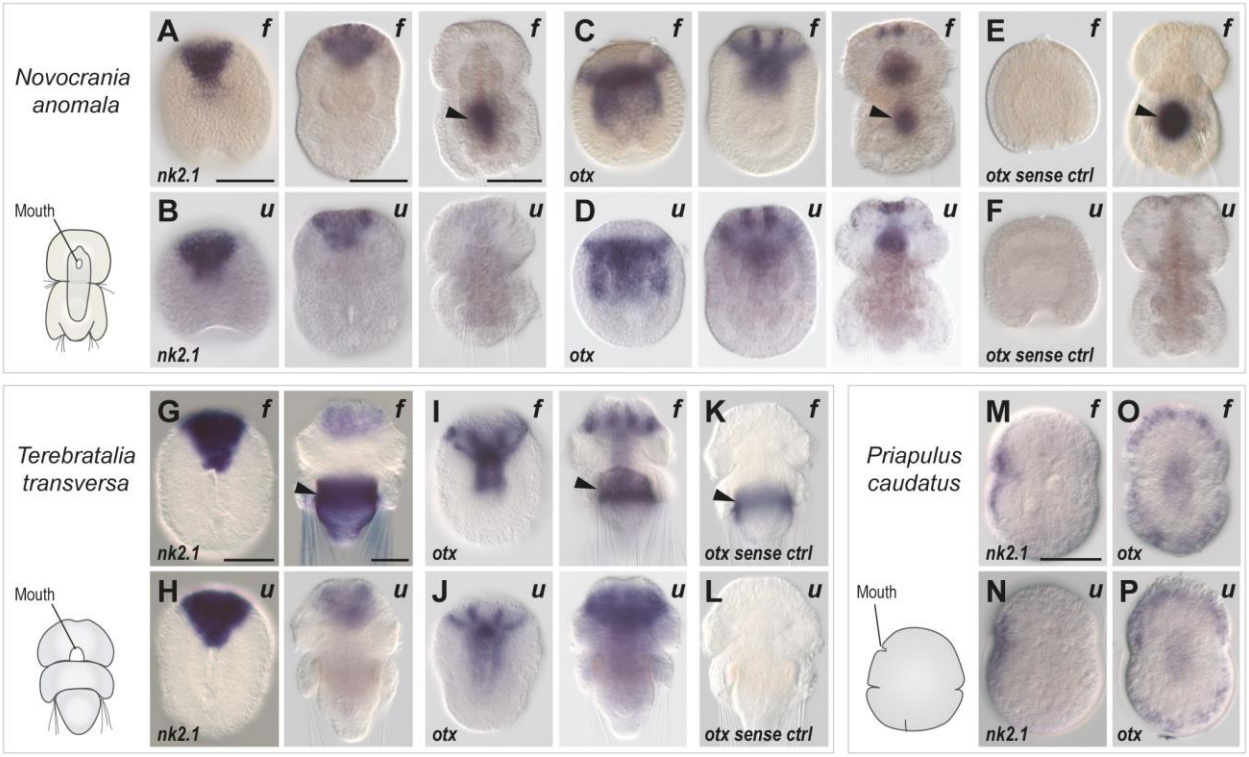

Figure 3. In situ hybridization on developmental stages of two brachiopods and a priapulid worm using either formamide (f) or urea-based (u) methods. A-F *Novocrania anomala*, G-L *Terebretalia transversa*, M-P *Priapulus caudatus*. Antisense (A-D, G-J, M-P) and sense control (E, F, K, L) probe gene names indicated on the bottom left of each panel. Developmental stages from left to right: (A-D) early gastrula, late gastrula and late larva; (E-J): early gastrula and late larva; (K-L): late larva; M-P: late gastrula. In all drawings and images, embryos and larvae are oriented with the anterior pole on top. Black arrows indicate aspecific signal, frequently seen with the traditional formamide method in the shell gland of *Novocrania anomala* (right images in A, C, E) and *Terebretalia transversa* (right images in G, I, K) late larvae. Scale bars, 50 µm.

Table 1. Overview of properties and health risks of formamide, the most diffuse denaturing agent, and of urea, a safer alternative (source *www.ncbi.nlm.nih.gov/pccompound*).

	Formamide	***Urea***
Alternative chemical names	Carbamaldehyde; Methanamide; 75-12-7	Carbamide; Carbonyldiamide; 57-13-6
Molecular formula	CH_3NO or $HCONH_2$	NH_2CONH_2 or CH_4N_2O
CAS	75-12-7	57-13-6
Molecular weight	45.041 g/mol	60.056 g/mol
Water solubility	1.0e6 mg/l (at 25°C)	5,45e5 mg/L (at 25°C)
GHS classification	Danger	Warning
Effect of short term exposure	The substance is moderately irritating to the eyes and skin. The substance may cause effects on the central nervous system.	The substance is irritating to the eyes, skin and respiratory tract.
Effect of long term exposure	May cause toxicity to human reproduction or development.	Repeated or prolonged contact with skin may cause dermatitis.

Table 2. A survey of the recent reports focused on formamide-alternatives for in situ hybridization. Hybridization types: CISH- Chromogenic In Situ Hybridization, FISH- Fluorescent In Situ Hybridization, FIVH- Fluorescent In Vivo Hybridization, GISH - Genomic In Situ Hybridization.

Hybridization type	*Formamide-substitute*	*Sample type*	*Reference*
CISH	4X SSC	Mammal tissue (Fibromatosis nodules)	(Berndt et al., 1996)
FISH	Urea-NaCl	Bacteria (*Staphylococcus aureus*)	(Lawson et al., 2012)
CISH / FISH	4M urea	Mammal tissue (miRNA in mouse brain)	(Søe et al., 2011)
IQFISH (1 hour-hybridization FISH)	Ethylene carbonate, sulfolane, propylene carbonate, c-butyrolactone, 2-pyrrolidone, d-valerolactam.	Mammal tissue (Breast carcinoma, tonsil and colon tissue)	(Matthiesen and Hansen, 2012)
FIVH	4M urea	Bacteria (*Helicobacter pylori*)	(Fontenete et al., 2013)
GISH	0.02X SSC	Plants (*Prospero*, *Melampodium*)	(Jang and Weiss-Schneeweiss, 2015)
FIVH	0.5M urea	Bacteria (*Helicobacter pylori*)	(Fontenete et al., 2015)
FISH	4M urea	Bacteria (*Helicobacter pylori*)	(Fontenete et al., 2016)
FISH	NaCl-EtOH		Sahlgrenska University Hospital (Sweden) *www.subsport.eu*
FISH	10% dextran sulfate / 20% glycerol / 0.9% NaCl or KCl		Patent WO 1996031626 A1
ISH on paraffin-embedded sections	Chaotropic agents (selected from the group of urea, salts of guanidinium or guanidine)		Patent EP 2563935 A1

Supplementary Material_1
Additional information regarding probe synthesis.

- **Genes**

Gene name	Probe name	CDS Lenght	Accession number
Minicollagen 3/4a (C. hemisphaerica)	*mcol3-4a*	821 bp	EU024529
DRGX (Dorsal Root Ganglia Homeobox) (C. hemisphaerica)	*drgx*	1021 bp	LN611639
pp5 (C. hemisphaerica)	*RFamide*	1566 bp	KX496951
Six3/6 (C. hemisphaerica)	*six3/6*	1189 bp	LN611635
Nk2.1 (N. anomala)	*nk2.1*	978 bp	KF946068
Otx (N. anomala)	*otx*	807 bp	KF946066
Nk2.1 (T. transversa)	*nk2.1*	972 bp	KF946076
Otx (T. transversa)	*otx*	822 bp	HQ679622
Nk2.1 (P. caudatus)	*nk2.1*	715 bp	KP013757
Otx (P. caudatus)	*otx*	822 bp	JX430801

- **Probe synthesis (*Clytia hemisphaerica*)**

Probes used in this study were synthesized from cDNA clones retrieved from our EST collection, and the DIG-labeled RNA probes were synthesized using the T7 RNA polymerase kit from Promega (according to vector and orientation of insert, SP6 or T3 polymerases could be necessary), purified using the ProbeQuant G-50 MicroColumns (GE Healthcare) and stored at -20°C.

For probe synthesis, add in order:

		Notes
DIG labelling mix RNA (Roche, #11277073910)	2 µl	10X solution with: 10 mM ATP, CTP, GTP (each), 6.5 mM UTP, 3.5 mM DIG-11-UTP.
RNasin Ribonuclease Inhibitor (Promega, #N2111)	0.5 µl	Protects RNA from degradation by RNases A, B and C.
DTT (Promega, #P1171)	2 µl (stock 100mM)	Dithiothreitol, necessary for the correct functioning of the ribonuclease inhibitor.
Transcription Optimized 5X Buffer (Promega, #P1181)	4 µl	
DNA template (1-2 µg)	*To calculate*	
RNase-free H_2O	*To volume*	
T7 RNA polymerase (Promega, #9PIP207)	0.5 µl	DNA-dependent RNA polymerase.
Total volume	20 µl	

- Let the synthesis run for 2-5 hours at 37°C, then stop the reaction by digesting the DNA template: add 1.5 µl of RQ1 RNase-free DNase (Promega, #M6101) and incubate at 37°C for 30 minutes.

- Add 30 µl of RNase free H_2O, and purify using the illustra ProbeQuant G-50 Micro Columns (GE Healthcare, #28-9034-08).

- Check 1 µl on 1% agarose gel, and quantify (with a nanodrop). Add 50 µl of deionized formamide (Molecular Biology, Biosolve BV, # 06812335) for storage at -20°C.

- **Probe synthesis (brachiopods and priapulid)**

Genes were cloned into the pGEM-T Easy vector (Promega), and probes were synthesized using the MEGAscript SP6 kit or the MEGAscript T7 kit (Invitrogen, Thermo Fisher Scientific), using the DIG-labeled nucleotides Digoxigenin-11-UTPs (Roche Diagnostics).

Supplementary Material_2
Detailed protocol for *Clytia hemisphaerica*.

	Step	*Solution*	*Duration*	*Temperature*	*Comment*
☐	Relaxation		10 min	On ice	
☐	Fixation *(CISH)*	3.7% formaldehyde plus 0.4% glutaraldehyde in 1X PBS (Phosphate-Buffered Saline, pH 7.4)	2 hours	On ice	Pre-chilled solution.
☐	Fixation *(FISH)*	3.7% formaldehyde in HEM buffer (0.1M HEPES pH 6.9, 50mM EGTA pH 7.20, 10mM $MgSO_4$)	36 hours	18°C	Renew solution once.
☐	Wash	1X PBST (1X PBS plus 0.1% Tween-20)	5 x 10 min	On ice	
☐	Wash	50% 1X PBST / 50% Methanol	1 x 10 min	On ice	
☐	Wash	100% Methanol	1 x 10 min	On ice	
☐	Storage	100% Methanol		-20°C	Can be directly re-hydrated.
☐	Wash	50% Methanol / 50% 1X PBST	1 x 5 min	RT	
☐	Wash	1X PBST	3 x 5 min	RT	
☐	Acetylation step	0.1M Triethanolamine (TEA) in 1X PBST	5 min	RT	Optional step.
☐	Acetylation step	0.25% Acetic anhydride in (0.1M TEA in 1X PBST)	5 min	RT	Optional step.
☐	Wash	1X PBST	10 min	RT	Optional step.
☐	Transfer	50% Hybridization Buffer (4M urea, 5X SSC, 1% dextran, 50 µg/ml of tRNA, 50 µg/ml of heparin, 1% SDS, and milliQ H_2O) / 50% 1X PBST	1 X 10 min	RT	
☐	Transfer	100% Hybridization buffer	20 min	RT	
☐	Pre-hybridization	100% Hybridization buffer	2 hours minimum	58°C	Pre-hybridizing overnight can improve the results.
☐	Hybridization	Probe in hybridization buffer	48-72 hours	58°C	Heat probe mix at 95°C for 5 min. Spin down and quickly apply probe, without letting it cool down.
☐	Stringent wash	4M urea, 0.1% Tween-20, 5X SSC, milliQ H_2O	3 x 30 min	58°C	

☐	Stringent wash	2M urea, 0.1% Tween-20, 2X SSC, milliQ H_2O	3 x 30 min	58°C	
☐	Stringent wash	0.1% Tween-20, 2X SSC, milliQ H_2O	2 x 30 min	58°C	
☐	Wash	MABT buffer (Maleic Acid Buffer, containing 0.1% Tween-20)	2 X 20 min	RT	
☐	Block	Blocking buffer (1% blocking solution, in MABT buffer)	1 hour	RT	
	CISH detection				
☐	Antibody incubation	Anti-DIG-AP (1:2000, in blocking buffer)	Overnight	4°C	
☐	Wash	MABT buffer	6 x 15 min	RT	
☐	Wash	NTMT *minus* buffer (NaCl, Tris-HCl at pH 9.5, Tween-20)	3 x 10 min	RT	Optional.
☐	Wash	NTMT buffer (NaCl, Tris-HCl at pH 9.5, $MgCl_2$, Tween-20)	2 x 10 min	RT	
☐	Colour development	NBT/BCIP in NTMT buffer (0.08 mg/ml of Nitro Blue Tetrazolium and 0.1 mg/ml of 5-Bromo-4-Chloro-3-Indolyl-Phosphate)	Until appropriate	RT/4°C	
☐	Rinse	milliQ H_2O		RT	
☐	Post-fixation	3.7% formaldehyde in 1X PBS	1 x 30 min	RT	
☐	Wash	1X PBS			
☐	Clarify & Mount	50% 1X PBS/ 50% glycerol			
☐	Clarify & Mount	70% glycerol			
	FISH detection				
☐	Antibody incubation	Anti-DIG-POD (1:2000, in blocking buffer)	Overnight	4°C	
☐	Wash	Reaction buffer (0.0015% H_20_2 in 1x PBS)	2 x 30 min	RT	Freshly prepared.
☐	Signal development	Fuorophore-conjugated tyramide kit (1:400, in reaction buffer)	1 hour	RT	Keep in dark.
☐	Rinse	1X PBS		RT	Keep in dark.
☐	Nuclear staining	Hoechst 33528 (in 1X PBS)	30 minutes	RT	Keep in dark.
☐	Rinse	1X PBS		RT	Keep in dark.
☐	Storage and mount	Citifluor AF-1		RT, then 4°C	Keep in dark.

Note: the urea buffer tends to be more viscous, and more prone to evaporation. We recommend sealing the samples carefully.

- **Main reagents**

	Company	#
Hybridization buffer		
Formamide – deionized. Molecular Biology	Biosolve BV; The Netherlands	06812335
Urea	Sigma	U5378
Dextran sulfate sodium salt, Mr ~200''00	Sigma	67578-25G
tRNA, from baker's yeast	Sigma	10109509001
Heparin sodium	Thermofisher	10239840
Antibody		
Anti-Digoxigenin-AP, Fab fragments *AP = Alkalyne Phosphatase*	Roche Applied Sciences (Sigma- Aldrich)	11093274910
Anti-Digoxigenin-POD, Fab fragments *POD = Horseradish Peroxidase*	Roche Applied Sciences (Sigma- Aldrich)	11207733910
Colour reaction		
BCIP/NBT Color Development Substrate	Promega	S3771
TSA Plus Fluorescence Amplification kit Cyanine 3/5	Perkin Elmer; Waltham, MA	NEL752001KT

Supplementary Material_3
Detailed protocol for brachiopod and priapulid species.

In situ hybridization protocol for: *T. transversa, N. anomala* and *P. caudatus.*

	Step	*Solution*	*Duration*	*Temperature*	*Comment*
☐	Relaxation	7.4% $MgCl_2x6H_2O$ in sea water	10- 15 min	Room temperature (RT)	
☐	Fixation	4% paraformaldehyde (PFA) in 1X PBST (1X PBS plus 0.1% Tween-20)	1 hour	RT	
☐	Wash	1X PBST	7 x 5 min	RT	
☐	Storage	Methanol	Minimum 24-48 hours	-20°C	Can be stored up to several months.
☐	Wash	1X PBST	5 x 5 min	RT	
☐	Permeabilize	10 µg/ml proteinase K in 1X PBST	Species/ stage specific*[1]	RT	No shaking.
☐	Arrest of digestion	2 mg/ml glycine in 1X PBST	2 x 5 min	RT	
☐	Wash	1% Triethanolamine (TEA) in 1X PBST	5 min	RT	
☐	Wash	Acetic anhydride *[2] in 1% TEA in 1X PBST	5 min	RT	
☐	Wash	Acetic anhydride *[3] in 1% TEA in 1X PBST	5 min	RT	
☐	Wash	1X PBST	2 x 5 min	RT	
☐	Fixation	3.7% formaldehyde in 1X PBST (+ 0.2% glutaraldehyde*[4])	1 hour	RT	Depends on hybridization buffer*[4].
☐	Wash	1X PBST	3 x 5 min	RT	
☐	Heating	1X PBST	10 min	80°C	
☐	Wash	1X PBST	5 min	RT	
☐	Transfer	Hybridization buffer *(see legend for recipe)*	5 min	RT	
☐	Transfer	Hybridization buffer	10 min	RT	
☐	Pre-hybridization	Hybridization buffer	Overnight	67°C	Hybridization temperature, and probe concentration need to be adjusted according to species and probe.*[5]
☐	Hybridization	Probe in hybridization buffer	24- 72 hours	67°C	
☐	Wash	Hyb-wash buffer (see legend for recipe)	15 min	67°C	
☐	Wash	Hyb-wash buffer	45 min	67°C	
☐	Stringent wash	75% Hyb-wash buffer / 25% 2X SSC	30 min	67°C	
☐	Stringent wash	50% Hyb-wash buffer / 50% 2X SSC	30 min	67°C	
☐	Stringent wash	25% Hyb-wash buffer / 75% 2X SSC	30 min	67°C	

☐	Stringent wash	2X SSC	30 min	67°C	
☐	Stringent Wash	0.2X SSC	3 x 20 min	67°C	
☐	Stringent Wash	75% 0.2X SSC / 25% 1X PBST	10- 15 min	RT	
☐	Wash	50% 0.2X SSC / 50% 1X PBST	10- 15 min	RT	
☐	Wash	25% 0.2X SSC / 75% 1X PBST	10- 15 min	RT	
☐	Wash	1X PBST	10- 15 min	RT	
☐	Wash/Block	1x PBSTx (1X PBS + 0.2% Triton-X100 + 0.1% BSA (Bovine Serum Albumine))	2 x 15 min	RT	
☐	Rinse	Maleic acid buffer	5 min	RT	
☐	Block	Blocking buffer (Boehringer-Mannheim blocking buffer, in maleic acid buffer)	1 hour	RT	
☐	Antibody incubation	Anti-DIG-AP (1:5000) in blocking buffer	Overnight	4°C	
☐	Wash	1X PBSTx	5 x 15 min	RT	
☐	Wash	1X PBST	5 x 30 min	RT	
☐	Wash	NTMT *minus* buffer (NaCl, Tris-HCl at pH 9.5, Tween-20)	3 x 10 min	RT	
☐	Wash	NTMT buffer (NaCl, Tris-HCl at pH 9.5, $MgCl_2$, Tween-20)	2 x 10 min	RT	
☐	Colour development	NBT/BCIP in NTMT buffer	Until necessary	RT	
☐	Wash	NTMT *minus* buffer	2 x rinse	RT	
☐	Wash	100% ethanol	2 x 5 min	RT	
☐	Wash	100% ethanol	20 min	RT	
☐	Wash	50% ethanol / 50% 1X PBST	5 min	RT	
☐	Wash	1X PBST	3 x 5 min	RT	
☐	Clarify & Mount	70% glycerol / 30% 1X PBST	Until appropriate	4°C	

*[1] 10 min *P. caudatus*, 9 min *T. transversa*
*[2] 1.5 µl acetic anhydride per 500 µl 1X PBST/TEA
*[3] 4.5 µl acetic anhydride per 500 µl 1X PBST/TEA
*[4] Glutaraldehyde is only necessary for urea-containing buffer, not for the formamide-based buffer.
*[5] In the case of *T. transversa* and *P. caudatus,* for *nk2.1* and *otx*, we incubated at 66°C and used probes at a concentration of 1 ng/µl.

Note: the urea buffer tends to be more viscous, and more prone to evaporation. We recommend sealing the samples carefully.

- **Recipes**

Hybridization buffer (10ml)

20X SSC	2.5 ml
Dextran	0.1 g
Urea	2.4 g
SDS (20%)	500 µl
Heparin (20 mg/ml)	25 µl
Salmon sperm, single stranded nucleic acid	50 µl
mQ H_2O	5.1 ml

Note: The traditional buffer includes 50% volume of deionized formamide (ultra pure, from VWR).

Hyb-Wash buffer (10 ml)

20X SSC	2.5 ml
Urea	2.4 g
Tween-20	10 µl
mQ H_2O	To volume

Note: The traditional buffer includes 50% volume of deionized formamide (ultra pure, from VWR).