A technical guide to fluorescence *in situ* hybridization

Alexander P Young [Corresp., 1], **Daniel J Jackson** [2], **Russell C Wyeth** [1]

[1] Department of Biology, St. Francis Xavier University, Antigonish, NS, Canada

[2] Geobiology, Georg-August Universität Göttingen, Göttingen, Germany

Corresponding Author: Alexander P Young
Email address: ayoung@stfx.ca

RNA-fluorescence *in situ* hybridization (FISH) is a powerful tool to visualize target mRNA transcripts in cultured cells, tissue sections or whole mount preparations. As the technique has been developed over time, an ever-increasing number of divergent protocols have been published. There is now a broad selection of options available to facilitate proper tissue preparation, hybridization, and post-hybridization background removal. Thus, a variety of considerations can affect the selection of reagents to achieve optimal results. Here we review the technical aspects of RNA-FISH, examining the most common methods associated with different sample types including cytological preparations and whole mounts. We discuss the application of commonly used reagents for tissue preparation, hybridization, and post-hybridization washing and provide explanations of the functional roles for each reagent. We also summarize probe selection and necessary controls for providing robust gene expression evidence. Taken together, this information will help the methods development process for investigators that seek to perform FISH in organisms without documented or optimized protocols.

A technical guide to RNA fluorescence *in situ* hybridization

Alexander P. Young[1]*, Daniel J. Jackson[2], Russell C. Wyeth[1]

[1]Department of Biology, St. Francis Xavier University, Antigonish, NS, Canada

[2]Department of Geobiology, Georg-August University of Göttingen, Göttingen, Germany

***Corresponding author**

Alexander P. Young
2320 Notre Dame Ave., Antigonish, Nova Scotia, B2G 1Z3, Canada
Email address: ayoung@stfx.ca

Abstract

RNA-fluorescence *in situ* hybridization (FISH) is a powerful tool to visualize target mRNA transcripts in cultured cells, tissue sections or whole mount preparations. As the technique has been developed over time, an ever-increasing number of divergent protocols have been published. There is now a broad selection of options available to facilitate proper tissue preparation, hybridization, and post-hybridization background removal. Thus, a variety of considerations can affect the selection of reagents to achieve optimal results. Here we review the technical aspects of RNA-FISH, examining the most common methods associated with different sample types including cytological preparations and whole mounts. We discuss the application of commonly used reagents for tissue preparation, hybridization, and post-hybridization washing and provide explanations of the functional roles for each reagent. We also summarize probe selection and necessary controls for providing robust gene expression evidence. Taken together, this information will help the methods development process for investigators that seek to perform FISH in organisms without documented or optimized protocols.

Introduction

Fluorescence *in situ* hybridization (FISH) is a powerful tool to visualize target DNA sequences or mRNA transcripts in cultured cells, tissue sections or whole mount preparations. FISH functions via the principles of nucleic acid thermodynamics whereby two complementary strands of nucleic acids readily anneal to each other under the proper conditions to form a duplex (RNA:RNA or DNA:DNA), known as a hybrid (Felsenfeld & Miles, 1967). Under energetically favourable conditions, strands of RNA and DNA can also anneal to form DNA:RNA hybrids (Rich, 1959, 1960; Milman, Langridge & Chamberlin, 1967). This phenomenon has facilitated the development of techniques that use either DNA or RNA probes to bind to DNA or RNA targets within a biological sample, a method broadly known as *in situ* hybridization (ISH). The earliest ISH protocols relied on radioactive probes that were costly, required long exposure times and were hazardous to the health of the investigator (Gall & Pardue, 1969; Pardue & Gall, 1969). Probes that relied on fluorophores instead of radioactive isotopes were later developed, these probes could be directly detected with fluorescence microscopy and the use of these probes became known as fluorescence *in situ* hybridization (FISH; Rudkin & Stollar, 1977). As FISH can be used to target DNA, modern FISH protocols are frequently used to identify positions of genes on chromosomes, diagnose diseases, and identify microorganisms (Kempf, Trebesius & Autenrieth, 2000; Wiegant et al., 2000; Hicks & Tubbs, 2005). However, FISH has also been developed to target RNA and thus visualize gene expression *in situ*, herein referred to as RNA-FISH (Singer & Ward, 1982). More recently, computational and imaging technology has further driven the development of RNA-FISH to allow for the visualization and automated quantification of individual messenger RNA transcripts (Femino et al., 1998; Levsky et al., 2002; Raj et al., 2008). The use of RNA-FISH to visualize individual mRNA molecules in this fashion

is currently known as single-molecule FISH (smFISH; Femino et al., 1998). Ultimately, there are several derivations of the original ISH method that have diverged to localize either DNA or RNA molecules with one of many detection methods. In this review, we focus on RNA-FISH methods.

As the number of techniques and applications for FISH has increased, the number of available reagents, probe types, and detection methods have also increased. The rise in protocol options has increased the complexity faced by a researcher when developing a new FISH protocol or attempting to adapt an established protocol for use with a non-conventional model organism. In particular, published protocols rarely make it clear which components are essential, technique- or species-specific, or "traditional" elements passed down from previous iterations of a protocol. The purpose of this review is to draw the common ISH variants and their rationales together and discuss the logic behind each step of the methodology to equip users with the knowledge to develop novel applications of RNA-FISH for sample types in which no established or optimized protocols exist. Thus, we present a broad survey of published RNA-FISH protocols to educate new users and streamline the methods development process for experienced investigators that seek to perform FISH in sample types without documented protocols.

Survey methodology

To compare differences in modern FISH methodologies (tissue preparation, hybridization, and post-hybridization), the literature was broadly surveyed using PubMed and Google Scholar to search terms including "FISH", "fluorescent", "fluorescence", and "*in situ* hybridization". We also cross-referenced each article to identify further relevant resources from the bibliography. Manuscripts that included sufficiently detailed methods were selected for comparison. Generally,

manuscripts from the last 10 years (after 2009) were preferred to reflect modern methods, however, we chose to also include seminal work that heavily influenced the development of the field. To support the discussion of the functionality of commonly used reagents, we searched for manuscripts that specifically explained the mechanistic underpinnings of the reagents.

The development of RNA-FISH and smFISH

The method of labeling strands of nucleic acids *in situ* has undergone substantial development since its inception by Gall & Pardue in 1969 (Figure 1). The earliest ISH techniques were documented in a pair of companion papers by Gall and Pardue (Gall & Pardue, 1969; Pardue & Gall, 1969). Gall & Pardue (1969) used RNA-based probes to label DNA in oocytes of the toad *Xenopus*. Pardue & Gall (1969) also used DNA-based probes to label DNA the same cell types from the same species. In both cases, these probes required autoradiography for visualization. The first fluorescence *in situ* detection of DNA with indirect immunofluorescence was performed by Rudkin & Stollar (1977). The authors used RNA probes with hapten-labeled nucleotides that could be targeted with rhodamine-labeled antibodies. When subsequently visualized with a fluorescence microscope, these circumvented many of the disadvantages associated with autoradiography (Bauman et al., 1980; Kislauskis et al., 1993). Rudkin & Stollar (1977) used the indirect immunofluorescence method successfully to label polytene chromosomes in *Drosophila melanogaster*. Direct fluorescent *in situ* detection (of DNA again) without the need for antibodies was later performed by Bauman et al. (1980). The authors labelled mitochondrial DNA in the insect trypanosome *Crithilia luciliae* using an RNA probe with rhodamine incorporated into the probe itself (RNA was oxidized with $NaIO_4$, coupled to tetramethyl rhodamine thio-semicarbazide and the labelled probe was phenol-chloroform

extracted). Although RNA-based probes had been used to this point, FISH had only been used to label DNA. Singer & Ward (1982) performed the first true RNA-FISH to visualize actin mRNA in a chicken muscle tissue culture. The authors used DNA probes labeled with biotin as a hapten (biotinated dUTP was incorporated into the DNA via nick-translation). Following hybridization, these probes were targeted first with primary antibodies and then with secondary rhodamine-conjugated antibodies to facilitate visualization. The secondary antibody labeling allowed Singer and Ward to achieve higher sensitivity and stronger fluorescence compared to the direct detection method of Bauman et al. (1980). Singer and colleagues later developed the method of so-called smFISH which could resolve individual mRNA transcripts (Femino et al., 1998). This smFISH method used probes that were directly labeled with five Cy3 molecules per probe molecule, and the fluorescence of each probe was calibrated to allow direct relation of signal quantity to hybrid quantity. Since these seminal studies, the applications of smFISH have expanded to visualize differential gene expression within heterogeneous cells (Raj et al., 2008; Taniguchi et al., 2010). The smFISH technique can also be multiplexed with several nucleic acid-based probes with different fluorophores to identify the expression of multiple genes within individual cells (Levsky et al., 2002; Raj & van Oudenaarden, 2009; Shalek et al., 2014). smFISH can also be paired with immunofluorescence or flow cytometry to simultaneously measure mRNA and protein abundance (Yoon, Pendergrass & Lee, 2016; Arrigucci et al., 2017).

In subsequent iterations of smFISH protocol development, the creation of a new probe type resulted in labelling that was refined to the point where automated quantification became possible. In the early development of RNA-FISH, probes had relied on either one fluorophore per probe molecule (and thus per hybridized transcript) or signal amplification via the

122 immunofluorescence methods. Both methods had issues producing signal-to-noise ratios that
123 could allow reliable quantification of transcript expression. With the development of smFISH,
124 Femino et al. (1998) improved the potential for quantification by using multiple fluorophores per
125 probe molecule, but the fluorophores within the probes were subject to interactions which
126 resulted in self-quenching. Consequently, an alternative type of probe to localize individual
127 mRNA molecules was developed by Raj et al. (2008) using multiple 20-mer oligonucleotides. A
128 series of short probes were designed to collectively span the length of the transcripts of interest
129 with each probe tagged by a single Alexa 594 fluorophore at the 3'-terminus to provide a
130 predictable number of fluorophores per transcript. The authors found that this approach was
131 more effective in labeling individual mRNA targets compared to traditional probes that spanned
132 the full length of the transcript. The method was highly sensitive and produced strong signal with
133 little variation and minimal background, such that individual mRNA transcripts could be
134 automatically counted with the companion software (Raj et al., 2008; Raj & van Oudenaarden,
135 2009; Lyubimova et al., 2013). The technology associated with the synthesis of multiple singly-
136 labeled probes has been patented and licensed to Biosearch Technologies Inc. (Petaluma, CA,
137 USA) where it has been made available under the brand name Stellaris® RNA FISH probes.
138
139 Here we review the technical aspects of RNA-FISH, including but not limited to smFISH. Based
140 on a critical analysis of published methods, we summarize the state of the technique with respect
141 to commonly used reagents for tissue preparation, hybridization, and post-hybridization washing
142 and provide explanations of the functional roles for each reagent. It is worth noting the
143 substantial overlap between many published ISH and FISH protocols with respect to tissue
144 preparation, hybridization, and post-hybridization. Thus, information has been drawn from a

broad selection of protocols and could also benefit the development of non-fluorescent (colormetric) ISH protocols (excluding the probe selection and detection process).

Technical aspects of FISH

Many permutations of the FISH methodology exist for a variety of niche purposes (Volpi and Bridger 2008). Despite the range of techniques available, there is a core method which is common among most: fixation (pre-hybridization), hybridization, and washing (post-hybridization). These processes are essential to a FISH protocol, and each requires specific reagents to be effective. Generally, the required reagents are similar for cytological, histological, and whole mount preparations. However, there are some differences which are highlighted below. Note that the design and synthesis of a probe against a DNA or RNA molecule of interest is also a critical phase of any ISH experiment, but we will not focus on this aspect here. However, characteristics such as the GC content, the propensity to form secondary structures, the overall length and specificity and probe quantity and quality must be considered.

Tissue preparation and permeabilization

Tissue preparation is one of the most critical aspects of a FISH protocol. Tissue preparation typically comprises both fixation and tissue permeabilization, and the balance of these is important in determining the degree of probe penetration as well as the morphological integrity of the sample. The most common fixatives are 4% formaldehyde or paraformaldehyde in phosphate buffered saline (PBS; Nakamura, Nakamura & Hamada, 2013; Neufeld et al., 2013; Kernohan & Bérubé, 2014; Shiura et al., 2014; Oka & Sato, 2015; Thiruketheeswaran, Kiehl & D'Haese, 2016). Formaldehyde is a crosslinking fixative that forms covalent links between

168 macromolecules such as lipids, peptides and DNA; this creates a mesh inside the cells or tissues
169 to hold their components in place and minimize enzymatic degradation over time (Eltoum et al.,
170 2001). Paraformaldehyde solutions produced from a powder will contain pure fixative, however,
171 paraformaldehyde will polymerize over time and become less effective as the polymers
172 precipitate from the solution (Thavarajah et al., 2012). Alternatively, commercial formalin
173 contains 37% formaldehyde in water and is supplemented with 10% methanol as a stabilizer.
174 Thus, a 1:10 dilution of commercial formalin solution is a common substitute for 4%
175 paraformaldehyde that does not require fresh preparation for each experiment (Thavarajah et al.,
176 2012).
177
178 Fixation protocols are generally consistent among cytological, histological, and whole mount
179 preparations, although whole mounts generally require longer treatments to ensure complete
180 penetration of the fixative. Fixation protocols generally consist of a treatment with 4%
181 paraformaldehyde (PFA) or formaldehyde in PBS for varied lengths of time and temperatures
182 (Table 1). These examples, and the link between sample size and "density" (larger and denser
183 samples need longer fixation) can provide some scope when estimating a fixation duration for
184 other sample types. Optimal fixation of planarian worms is achieved with 4% formaldehyde for
185 20 minutes (Pearson et al., 2009; Rink, Vu & Alvarado, 2011). For bacterial species or
186 eukaryotic cells, 4% PFA is used to fix cells for roughly 30 – 90 minutes (Wang et al., 2015;
187 Aistleitner et al., 2018; Cardinale et al., 2018; Rocha, Almeida & Azevedo, 2018). Fruit fly
188 (*Drosophila melanogaster*) embryos are typically fixed in 4% PFA for 20 – 30 minutes
189 (Hauptmann et al., 2016; Jandura et al., 2017; Szabo et al., 2018). Zebrafish (*Danio rerio*)
190 embryos and the annelid *Platynereis dumerilii* can be suitably fixed in 4% PFA for 2 hours

(Jékely & Arendt, 2007; Steinmetz et al., 2011) but can alternatively be fixed overnight at 4˚C (Oxtoby & Jowett, 1993; Lauter, Söll & Hauptmann, 2011a,b; Marra et al., 2017). Arms of the brittle star (*Amphiura filiformis*) are also fixed in 4% PFA overnight at 4˚C. Embryos of the brachiopods *Terebratalia transversa* and *Novocrania anomala* should be fixed in 4% formaldehyde for 4 hours (Schiemann et al., 2017; Gąsiorowski & Hejnol, 2019). The starlet sea anemone (*Nematostella vectensis*) and an acoelomorph worm (*Convolutriloba longifissura*) have been successfully fixed for ISH with 3.7% formaldehyde supplemented with 0.3% glutaraldehyde which is another strong cross-linking agent (Finnerty et al., 2003; Martindale, Pang & Finnerty, 2004; Hejnol & Martindale, 2008). Whole mouse brains are often fixed in 4% PFA for up to 6 hours at room temperature or overnight at 4˚C though fixation of brain tissue is recommended not to exceed 24 hours (Kernohan & Bérubé, 2014; Kasai et al., 2016; Lanfranco et al., 2017; Hua et al., 2018).

As an alternative to formaldehyde, some protocols employ an alcohol-based fixation method using either ethanol (Schurter, LeBrun & Harrison, 2002) or methanol (Legendre et al., 2013). Methanol will dehydrate tissues and strip the membrane lipids to improve permeability (Hoetelmans et al., 2001). Thus, if cross-linking is also desired, formalin may improve tissue permeability over paraformaldehyde due to the added methanol. For example, fixative solutions that contain alcohol and formaldehyde improve permeability in gram-positive bacterial preparations and may retain higher DNA quality in cytological preparations (Manz et al., 1994; Shaffer et al., 2013). Ethanol can also help to strip the external wax and lipids from plant tissues (Bleckmann & Dresselhaus, 2016).

214 Beyond the choice of fixative, fixation temperature can also have a substantial impact on the
215 final tissue quality (Fox et al., 1985; Thavarajah et al., 2012). With the use of formaldehyde, heat
216 can accelerate the fixation process; although heat also increases the release of formaldehyde
217 fumes which are hazardous to human health (Fox et al., 1985; Titford, 2001). For nucleic acid
218 visualization, reduced temperatures of 4˚C have been shown to preserve RNA throughout the
219 fixation process (Bussolati et al., 2011). Additionally, ice-cold solutions of alcohol fixatives are
220 recommended as the reduced temperatures will reduce the risk of over-permeabilization and
221 subsequent leakage of target molecules.
222
223 Following fixation, samples generally must be permeabilized to allow for proper penetration of
224 hybridization reagents. Detergent treatment of fixed tissue is commonly employed at a
225 concentration of 0.1% as it substantially improves permeability of the tissues via disruption of
226 cellular membranes. The use of Tween-20 is common but other detergents including sodium
227 dodecyl sulfate (SDS) and Triton X-100 can also be used. The detergent 3-[(3-
228 cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) is commonly used as an
229 additive detergent to multiplex FISH with immunofluorescence as it effectively protects the
230 native structure of proteins (Meyer, Garzia & Tuschl, 2017; Sepsi et al., 2018). Whole mount
231 preparations generally require stronger detergent treatments compared to cytological
232 preparations or sectioned tissue, thus, a more aggressive detergent treatment such as 4% Triton
233 X-100 can be effective in whole mounts (Croll et al., 1999).
234
235 Treatment with a nonspecific protease such as proteinase K will permeabilize the tissues after
236 fixation and can also release target nucleic acid molecules from bound proteins (such as RNA

binding proteins) making them more accessible for hybridization. There is generally an inverse relationship between fixation time and the strength of the proteinase treatment as more highly fixed tissues will require a stronger protease digestion to become permeable to the probe. A protease treatment is not always necessary for bacterial or eukaryotic cells as a detergent is usually sufficient, however, a brief treatment with a dilute solution of proteinase K should be considered if probe penetration is the suspected cause of an issue (Carr et al., 2005). Zebrafish embryos are treated with 10 µg/mL proteinase K in PBST for 2 – 20 minutes depending on the age, the same treatment is recommended for fruit fly and snail embryos as well as whole mount planarian worms (Oxtoby & Jowett, 1993; Pearson et al., 2009; Hauptmann et al., 2016; Jackson, Herlitze & Hohagen, 2016). Some protocols call for brain sections to be treated with proteinase K, however, many protocols omit this step as permeability is less of an issue with sectioned material (Kasai et al., 2016; Hua et al., 2018). The proteinase K treatment will require careful optimization as too little digestion will prevent probe penetration whereas too much digestion will destroy the morphology of the tissue and lead to increased background (Tessmar-Raible et al., 2005; Bleckmann & Dresselhaus, 2016). Both the optimal proteinase K concentration and duration of treatment will vary among tissue types. After proteinase K treatments, tissues can be post-fixed for 10 – 30 minutes to preserve morphology throughout the hybridization process (Tessmar-Raible et al., 2005; Jackson, Herlitze & Hohagen, 2016).

Further permeabilization treatments are available as an alternative or an addition to protease treatments. A treatment of 1 M HCl at 37˚C for 30 – 50 minutes is effective to improve permeability of mycolic-acid-containing bacterial cells whereas other bacteria (including *Escherichia coli*) can be permeabilized in only 10 minutes (Macnaughton, O'Donnell & Embley,

1994). The addition of Triton X-100 or other detergent directly to the fixative in the initial fixation protocol has also been used to improve the permeability of bacterial cells through its interaction with cell envelope lipid molecules (Jackson, Herlitze & Hohagen, 2016; Rocha, Almeida & Azevedo, 2018). Zebrafish embryos that are stored in methanol can be treated with 2% H_2O_2 for 20 minutes at room temperature to improve permeability (Lauter, Söll & Hauptmann, 2011b). This H_2O_2 treatment can also quench endogenous peroxidase activity and bleach tissues to reduce background in horseradish peroxidase-based assays (Marra et al., 2017). In the preparation of whole-mounts with particularly tough integument, a digestion with 0.25% collagenase can be incorporated to improve permeability of dermal layers (Wyeth & Croll, 2011). In our experience, careful optimization of the balance between fixation (strength, length and temperature thereof) and a proteinase based permeabilization is time well spent.

Hybridization

The purpose of hybridization is to create the optimal environment for the probe to bind to the RNA target. The hybridization reaction can contain an array of different components (Table 2). In addition to the tissue, most documented hybridization solutions comprise a saline-sodium citrate buffer (SSC) with formamide, vanadyl-ribonucleoside complex (VRC), dextran sulfate, bovine serum albumin (BSA), competitor tRNA or DNA, and the probe (Pinkel et al., 1988; Singer, 1998; Shaffer et al., 2013; Kernohan & Bérubé, 2014; Oka & Sato, 2015). Alternative components include Denhardt's solution, ethylenediaminetetraacetic acid (EDTA), and Tween-20 (Langenbacher et al., 2015; Parker et al., 2019). In addition to the recipe of the hybridization solution, there are several reaction conditions that must be considered, including salt concentration, and the temperature and duration of the hybridization reaction.

283

284 Formamide reduces the annealing temperatures of nucleic acid strands and allows the
285 hybridization to take place at lower temperatures, improving structural preservation of the tissue
286 (McConaughy, Laird & McCarthy, 1969; Bauman et al., 1980; Blake & Delcourt, 1996;
287 Fontenete et al., 2016). Formamide generally composes 50% of the final volume of the
288 hybridization buffer. Formamide is a toxic substance and, therefore, proper safety precautions
289 must be made to avoid inhalation and direct contact with formamide (Warheit et al., 1989).
290 Protocols that use safer alternatives to formamide, such as urea (Sinigaglia et al., 2018) have
291 been developed but have yet to gain popularity (Volpi, 2017).

292

293 VRC is an RNase inhibitor that is used protect RNA-based probes or targets from enzymatic
294 degradation (Berger & Birkenmeier, 1979; Frazier & Champney, 2012). VRC is typically added
295 to the hybridization buffer at a final concentration of 10 mM as a precautionary measure. VRC is
296 not compatible with solutions that contain EDTA as an equimolar concentration of a chelating
297 agent will inactivate the VRC (Puskas et al., 1982).

298

299 Dextran sulfate is an anhydroglucose polymer that absorbs water molecules to become hydrated
300 and reduce the free water in the reaction. This forces the probe and the target closer together, an
301 effect referred to as “molecular crowding”, which enhances the rate of hybridization of the probe
302 to the target as it increases the effective probe concentration (Lederman, Kawasaki & Szabo,
303 1981). Dextran sulfate can also improve fluorescent signals (van Gijlswijk et al., 1996; Franks et
304 al., 1998). Dextran sulfate is a synthetic analogue of heparin which can also be used in the
305 hybridization buffer and has also been reported to reduce background signal (Singh & Jones,

1984). Dextran sulfate is most often employed at a concentration of 50 to 100 mg/mL (Singer & Ward, 1982; Oka & Sato, 2015; Parker et al., 2019).

BSA is used as a blocking agent to reduce background signal and improve the visibility of the probe (Choo, 2008). BSA blocks nonspecific binding of probe molecules to nucleic acid binding sites on proteins within the tissue as it saturates the binding sites prior to the introduction of the probe. The use of BSA as a blocking agent may be especially important when using antibodies conjugated with a fluorophore. BSA is generally used at a concentration of 1 mg/mL (Thiruketheeswaran, Kiehl & D'Haese, 2016) up to 10 mg/mL (Singer & Ward, 1982)

Finally, competitor DNA or tRNA is generally included in the hybridization buffer. Typically, sheared salmon sperm DNA or tRNA from *E. coli* or yeast is used. The purpose of competitive nucleic acids is to saturate nonspecific binding sites for probes to reduce background. Additionally, the competitor tRNA may protect target mRNA molecules via nonspecific blocking of RNase molecules that may have contaminated the solution. The optimal concentration of tRNA within the hybridization buffer should be empirically determined as it may vary widely depending on the tissue sample and the probe (Langenbacher et al., 2015; Liu et al., 2019).

There are several alternative hybridization buffer components that can be used to facilitate an optimal hybridization environment. Denhardt's solution is a broad blocking reagent composed of BSA, Ficoll® type 400 and polyvinylpyrrolidone that can be used compared to BSA alone. EDTA is a chelating agent that can be added to a final concentration of 10 mM to remove free

329 divalent ions such as magnesium. As EDTA can inactivate the VRC, both components cannot be
330 used together. However, EDTA has been reported to inactivate RNases as well which could
331 render the VRC unnecessary (Puskas et al., 1982).
332
333 When the reagent recipe has been established to create a supportive hybridization solution, the
334 hybridization conditions must also be determined to facilitate optimal hybridization. We believe
335 attention should be first given to the following three parameters regarding hybridization: salt
336 concentration; hybridization temperature; and duration of hybridization. Optimal hybridization
337 will occur under conditions that allow the hybridization of the probe to the target but prevent the
338 formation of nonspecific hybrids. Conditions that only promote the sole formation of highly
339 stable hybrids are known as highly stringent conditions whereas more permissive conditions that
340 may allow the formation of nonspecific hybrids are considered less stringent. The stringency of
341 the hybridization is affected by the concentration of salt in the hybridization solution (lower
342 concentrations are more stringent) as well as the hybridization temperature (higher temperatures
343 are more stringent). It is most common to keep the salt concentration constant (750 mM NaCl,
344 87.5 mM sodium citrate) and simply adjust the hybridization temperature to achieve the ideal
345 stringency (Pearson et al., 2009; Jackson, Herlitze & Hohagen, 2016). An initial denaturation
346 step of 75˚C for 10 minutes is effective to denature all target and probe RNA and maximize
347 surface area of the probe and the target to facilitate hybridization, the sample would then be
348 immediately adjusted to the designated hybridization temperature (Jékely & Arendt, 2007;
349 Jackson, Herlitze & Hohagen, 2016). The optimal hybridization temperature is dependent on the
350 length and composition of the probe with higher temperatures being more stringent and less
351 conducive to hybridization. Although hybridization temperature should be empirically optimized

for every probe individually, short oligonucleotide probes (20-50 nucleotides) generally require lower hybridization temperatures of 37˚C whereas longer riboprobes of 1000+ nucleotides are able to hybridize at temperatures >55°C (Pearson et al., 2009; Jackson, Herlitze & Hohagen, 2016; Fontenete et al., 2016). Generally, the hybridization step cannot be over-incubated and usually involves determining at which point labelling no longer improves. Thus, an extended hybridization should be performed to allow probes to completely occupy available targets. Generally, 12-24 hours is sufficient, regardless of the probe type (Carleton et al., 2014; Jackson, Herlitze & Hohagen, 2016; Meyer, Garzia & Tuschl, 2017; Jandura et al., 2017). Ultimately, salt concentration, hybridization temperature, and hybridization duration can be adjusted to create the optimal hybridization conditions, with enough stringency to get only specific labelling, while maintaining the strongest possible labelling intensity. To streamline optimization, the salt concentration and hybridization duration can be held constant while the hybridization temperature is carefully optimized to the probe.

Post-hybridization treatments

The purpose of the post-hybridization washes is to separate nonspecific hybrids and remove unbound probe molecules from the tissue to minimize background signal. Samples are typically subjected to increasingly stringent washes in SSC buffer containing formamide and a detergent (Table 3; Jackson, Herlitze & Hohagen, 2016; Thiruketheeswaran, Kiehl & D'Haese, 2016). Typically, the increased stringency is achieved through sequential washes with incrementally reduced salt concentrations while the wash temperature is matched to the hybridization temperature (Martindale, Pang & Finnerty, 2004; Hejnol & Martindale, 2008; Jackson, Herlitze & Hohagen, 2016; Schiemann et al., 2017; Gąsiorowski & Hejnol, 2019). At the end of washing,

the goal is to have only the most specific and stable hybrids remaining. A wash progression ending with a higher concentration of salt (or at a lower temperature, i.e. lower stringency) will be less likely to denature and remove nonspecific hybrids, but also may preserve greater intensity of specific labelling.

In addition to nonspecific hybrids, autofluorescence and excessive background are issues that can diminish the visibility of true signal and influence the interpretation of the results. Treatment with 0.1% Sudan Black B in 70% ethanol is effective to minimize autofluorescence in sectioned brain tissue as well as cultured cells (Oliveira et al., 2010; Qi et al., 2017). If background signal is an issue, tissues can be acetylated with 0.3% acetic anhydride in triethanolamine for 5 – 10 minutes (Jackson, Herlitze & Hohagen, 2016). This acetylation neutralizes positively charged proteins in the tissue that could otherwise engage in electrostatic interactions with negatively charged probes.

Probe selection and optimization for FISH

Probes are nucleic acid strands that may be composed of DNA, cDNA or RNA; they may be single-stranded or double-stranded and may vary in length from 20 bases to over 1500 bases. Regardless of the probe type, the sequence of the probes must be complementary to the target sequence to ensure proper hybridization. Probes can be modified with a fluorophore directly attached to the probe to be detectable with fluorescence microscopy. Fluorophores may also be bound to an antibody that binds to an antigen that is incorporated into the probe which is known as secondary detection.

RNA-based riboprobes targeting full transcripts of upwards of 1500 bases are still common as they are simple to produce in the absence of DNA-synthesis technology. Riboprobes are typically prepared through *in vitro* transcription of a target sequence that has been cloned and then prepared as a purified PCR product. Thus, cloned sequences with flanking RNA polymerase promoters can be used with an appropriate RNA polymerase to produce RNA probes. Secondary detection is most common with riboprobes as nucleotides tagged with hapten molecules such as digoxigenin that are easily incorporated into the transcription reaction. The hapten molecules in the transcribed probe are then subsequently targeted by fluorophore-bound antibodies. One advantage of riboprobes (rather than DNA-based probes) is that an RNase treatment can follow the post-hybridization step to reduce background. This is only appropriate with riboprobes as RNA:RNA hybrids are unaffected by RNases whereas DNA:RNA hybrids will be degraded (Keller & Crouch, 1972; Donà & Houseley, 2014). Note, however, that unintentional RNase contamination earlier in the protocol will be detrimental as single-stranded riboprobes are sensitive to RNases prior to hybridization.

The other prominent probe type in modern FISH protocols is the oligonucleotide probe – a cocktail of short single-stranded synthetic DNA probes that collectively span the length of the target (Figure 2; Femino et al., 1998; Raj et al., 2008; Zenklusen & Singer, 2010). Each individual probe molecule can be labeled with a fluorophore on the 5’ end, 3’ end, or both. A broad selection of fluorophores are available including Cy3, Cy5, Alexa fluor (Invitrogen), and Quasar (LGC Biosearch Technologies) depending on the desired absorption/emission spectra, budget, or personal preference. Oligonucleotide probes can be advantageous for particularly challenging tissues as the small probes can penetrate the tissue more efficiently. Furthermore, as

each oligonucleotide probe binds to the target, the transcript will relax and facilitate the hybridization of additional probe molecules (Baker, 2012). Oligonucleotides also have the highest specificity possible as they are less tolerant of mismatches that lead to nonspecific binding (Hougaard, Hansen & Larsson, 1997; Insam, Franke-Whittle & Goberna, 2009).

Controls for an *in situ* hybridization experiment

An often overlooked aspect of FISH experiments is how to employ controls to detect false positive results, and to ensure that staining patterns represent genuine biological signals. If a staining pattern is observed following a FISH experiment, it could mean that the hybridization was successful, but it could also mean that the probe bound non-specifically to an off-target sequence. Additionally, a lack of observable signal could mean that the target is not expressed, but it may also indicate an issue with the protocol despite the presence of the target. We would encourage creativity in carefully designing control experiments to identify the causes of undesired or absent results. Some potential control treatments are suggested below.

There are several positive controls that can potentially be used to verify both the efficacy of the FISH protocol and the expected behaviour of all reagents. One type of positive control probe is a poly(dT) probe which can verify that the fixed sample and RNA preservation is of sufficient quality and that the protocol works effectively. The poly(dT) probe will detect poly-A tails of any mRNA molecule, therefore a negative signal from a poly(dT) probe is indicative of poor tissue quality or some other fundamental problem that impedes hybridization (Sarkar & Hopper, 1998). An informative counter control to this probe would be a poly(dA) probe where a relatively reduced signal would be expected. A more specific positive control to verify basic

444 protocol function is to use a probe against a constituently expressed gene such as actin or tubulin
445 that would ideally also be expected to give a spatially discrete staining pattern (Oschwald,
446 Richter & Grunz, 1991; Kaplan et al., 1992). Considering the relative ease and low cost of
447 generating transcriptome data nowadays it is feasible to also select genes with high levels of
448 expression for use as positive controls in the tissue or developmental stage of interest. Finally, if
449 no signal can be generated *in situ* with a positive control it may be informative to perform a
450 simple *in vitro* dot blot. By spotting a diluted series of the probe onto a membrane and detecting
451 these spots with the same reagents used in the *in situ* experiment any technical problems arising
452 from the reagents can be ruled out or quickly identified.
453
454 Conversely, negative controls can identify nonspecific probe binding for direct labelling and
455 nonspecific antibody binding for indirect labelling experiments. Parallel treatments in which one
456 sample has been pre-treated with RNase will also indicate if the probe is binding exclusively to
457 RNA (no signal is expected in the RNase treated sample). A similar treatment with DNAse will
458 identify any binding to DNA. By omitting the antibody from indirect detection-based
459 experiments, non-specific cross-reactivity of the antibody can be identified. A sense probe can
460 also be used in parallel with the normal antisense probe. A sense probe should not form a hybrid
461 within the fixed tissue as it will not be complementary to a target, and thus can only produce
462 non-specific binding. If sense and antisense probes are used in parallel and only the antisense
463 probe produces a signal, and all other controls are also verified, it is likely that the probe is
464 specific and hybridized to the desired mRNA target (Piette et al., 2008). While this type of
465 control is commonly employed in the literature and requested by reviewers, it has been reported
466 that some genes are transcribed from both the sense and anti-sense DNA strands (Katayama et

al., 2005; Zhang et al., 2006; Hongay et al., 2006; Finocchiaro et al., 2007). In our experience we find a combination of the above controls and more importantly, experience with a range of probes against different genes, will quickly give the user a sense of what is a general non-specific background versus a genuine biological signal.

References

Aistleitner K, Jeske R, Wölfel R, Wießner A, Kikhney J, Moter A, Stoecker K. 2018. Detection of Coxiella burnetii in heart valve sections by fluorescence *in situ* hybridization. *Journal of Medical Microbiology* **67**:537–542. DOI: 10.1099/jmm.0.000704.

Arrigucci R, Bushkin Y, Radford F, Lakehal K, Vir P, Pine R, Martin D, Sugarman J, Zhao Y, Yap GS, Lardizabal AA, Tyagi S, Gennaro ML. 2017. FISH-Flow, a protocol for the concurrent detection of mRNA and protein in single cells using fluorescence *in situ* hybridization and flow cytometry. *Nature Protocols* **12**:1245–1260. DOI: 10.1038/nprot.2017.039.

Baker M. 2012. RNA imaging *in situ*. *Nature Methods* **9**:787–790. DOI: 10.1038/nmeth.2108.

Bauman JG, Wiegant J, Borst P, van Duijn P. 1980. A new method for fluorescence microscopical localization of specific DNA sequences by *in situ* hybridization of fluorochromelabelled RNA. *Experimental Cell Research* **128**:485–490.

Berger SL, Birkenmeier CS. 1979. Inhibition of intractable nucleases with ribonucleoside--vanadyl complexes: isolation of messenger ribonucleic acid from resting lymphocytes. *Biochemistry* **18**:5143–5149.

Blake RD, Delcourt SG. 1996. Thermodynamic effects of formamide on DNA stability. *Nucleic Acids Research* **24**:2095–2103.

Bleckmann A, Dresselhaus T. 2016. Fluorescent whole-mount RNA *in situ* hybridization (F-WISH) in plant germ cells and the fertilized ovule. *Methods* **98**:66–73. DOI: 10.1016/j.ymeth.2015.10.019.

Bussolati G, Annaratone L, Medico E, D'Armento G, Sapino A. 2011. Formalin fixation at low temperature better preserves nucleic acid integrity. *PLOS ONE* **6**:e21043. DOI: 10.1371/journal.pone.0021043.

Cardinale M, Luvisi A, Meyer JB, Sabella E, De Bellis L, Cruz AC, Ampatzidis Y, Cherubini P. 2018. Specific fluorescence *in situ* hybridization (FISH) test to highlight colonization of xylem vessels by *Xylella fastidiosa* in naturally infected olive trees (*Olea europaea* L.). *Frontiers in Plant Science* **9**. DOI: 10.3389/fpls.2018.00431.

Carleton J, Lovell PV, McHugh A, Marzulla T, Horback K, Mello CV. 2014. An optimized protocol for high-throughput *in situ* hybridization of zebra finch brain. *Cold Spring Harbor protocols* 2014:1249–1258. DOI: 10.1101/pdb.prot084582.

Carr EL, Eales K, Soddell J, Seviour RJ. 2005. Improved permeabilization protocols for fluorescence *in situ* hybridization (FISH) of mycolic-acid-containing bacteria found in foams. *Journal of Microbiological Methods* **61**:47–54. DOI: 10.1016/j.mimet.2004.10.023.

Choo KHA. 2008. *In Situ Hybridization Protocols*. Springer Science & Business Media.

Croll RP, Voronezhskaya EE, Hiripi L, Elekes K. 1999. Development of catecholaminergic neurons in the pond snail, *Lymnaea stagnalis*: II. Postembryonic development of central and peripheral cells. *The Journal of Comparative Neurology* **404**:297–309.

Donà F, Houseley J. 2014. Unexpected DNA loss mediated by the DNA binding activity of ribonuclease A. *PLoS ONE* **9**. DOI: 10.1371/journal.pone.0115008.

Eltoum I, Fredenburgh J, Myers RB, Grizzle WE. 2001. Introduction to the theory and practice of fixation of tissues. *Journal of Histotechnology* **24**:173–190. DOI: 10.1179/his.2001.24.3.173.

Felsenfeld G, Miles H. 1967. The physical and chemical properties of nucleic acids. *Annual Review of Biochemistry* 36:407–448. DOI: 10.1146/annurev.bi.36.070167.002203.

Femino AM, Fay FS, Fogarty K, Singer RH. 1998. Visualization of single RNA transcripts *in situ*. *Science* **280**:585–590. DOI: 10.1126/science.280.5363.585.

Finnerty JR, Paulson D, Burton P, Pang K, Martindale MQ. 2003. Early evolution of a homeobox gene: the parahox gene Gsx in the Cnidaria and the Bilateria. *Evolution & Development* **5**:331–345. DOI: 10.1046/j.1525-142X.2003.03041.x.

Finocchiaro G, Carro MS, Francois S, Parise P, DiNinni V, Muller H. 2007. Localizing hotspots of antisense transcription. *Nucleic Acids Research* **35**:1488–1500. DOI: 10.1093/nar/gkm027.

Fontenete S, Guimarães N, Wengel J, Azevedo NF. 2016. Prediction of melting temperatures in fluorescence *in situ* hybridization (FISH) procedures using thermodynamic models. *Critical Reviews in Biotechnology* **36**:566–577. DOI: 10.3109/07388551.2014.993589.

Fox CH, Johnson FB, Whiting J, Roller PP. 1985. Formaldehyde fixation. *Journal of Histochemistry & Cytochemistry* **33**:845–853. DOI: 10.1177/33.8.3894502.

Franks AH, Harmsen HJM, Raangs GC, Jansen GJ, Schut F, Welling GW. 1998. Variations of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Applied and Environmental Microbiology* **64**:3336–3345.

Frazier AD, Champney WS. 2012. The vanadyl ribonucleoside complex inhibits ribosomal subunit formation in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **67**:2152–2157. DOI: 10.1093/jac/dks182.

Gall JG, Pardue ML. 1969. Formation and detection of RNA-DNA hybrid molecules in cytological preparations. *Proceedings of the National Academy of Sciences of the United States of America* **63**:378–383.

Gąsiorowski L, Hejnol A. 2019. Hox gene expression in postmetamorphic juveniles of the brachiopod *Terebratalia transversa*. *EvoDevo* 10. DOI: 10.1186/s13227-018-0114-1.

van Gijlswijk RP, Wiegant J, Raap AK, Tanke HJ. 1996. Improved localization of fluorescent tyramides for fluorescence *in situ* hybridization using dextran sulfate and polyvinyl alcohol. *Journal of Histochemistry & Cytochemistry* **44**:389–392. DOI: 10.1177/44.4.8601698.

Hauptmann G, Söll I, Krautz R, Theopold U. 2016. Multi-target chromogenic whole-mount *in situ* hybridization for comparing gene expression domains in *Drosophila* embryos. *Journal of Visualized Experiments : JoVE*. DOI: 10.3791/53830.

Hejnol A, Martindale MQ. 2008. Acoel development indicates the independent evolution of the bilaterian mouth and anus. *Nature* **456**:382–386. DOI: 10.1038/nature07309.

Hicks DG, Tubbs RR. 2005. Assessment of the HER2 status in breast cancer by fluorescence *in situ* hybridization: a technical review with interpretive guidelines. *Human Pathology* **36**:250–261. DOI: 10.1016/j.humpath.2004.11.010.

Hoetelmans RW, Prins FA, Cornelese-ten Velde I, van der Meer J, van de Velde CJ, van Dierendonck JH. 2001. Effects of acetone, methanol, or paraformaldehyde on cellular structure, visualized by reflection contrast microscopy and transmission

and scanning electron microscopy. *Applied immunohistochemistry & molecular morphology: AIMM* **9**:346–351.

Hongay CF, Grisafi PL, Galitski T, Fink GR. 2006. Antisense transcription controls cell fate in *Saccharomyces cerevisiae*. *Cell* **127**:735–745. DOI: 10.1016/j.cell.2006.09.038.

Hougaard DM, Hansen H, Larsson L-I. 1997. Non-radioactive *in situ* hybridization for mRNA with emphasis on the use of oligodeoxynucleotide probes. *Histochemistry and Cell Biology* **108**:335–344. DOI: 10.1007/s004180050174.

Hua R, Yu S, Liu M, Li H. 2018. A PCR-based method for RNA probes and applications in neuroscience. *Frontiers in Neuroscience* **12**. DOI: 10.3389/fnins.2018.00266.

Insam H, Franke-Whittle I, Goberna M. 2009. *Microbes at work: From wastes to resources*. Springer Science & Business Media.

Jackson DJ, Herlitze I, Hohagen J. 2016. A whole mount *in situ* hybridization method for the gastropod mollusc *Lymnaea stagnalis*. *Journal of Visualized Experiments*. DOI: 10.3791/53968.

Jandura A, Hu J, Wilk R, Krause HM. 2017. High resolution fluorescent *in situ* hybridization in *Drosophila* embryos and tissues using tyramide signal amplification. *Journal of Visualized Experiments : JoVE*. DOI: 10.3791/56281.

Jékely G, Arendt D. 2007. Cellular resolution expression profiling using confocal detection of NBT/BCIP precipitate by reflection microscopy. *BioTechniques* **42**:751–755. DOI: 10.2144/000112462.

Kaplan BB, Gioio AE, Capano CP, Crispino M, Giuditta A. 1992. beta-actin and beta-tubulin are components of a heterogeneous mRNA population present in the squid giant axon. *Molecular and Cellular Neurosciences* **3**:133–144.

Kasai A, Kakihara S, Miura H, Okada R, Hayata-Takano A, Hazama K, Niu M, Shintani N, Nakazawa T, Hashimoto H. 2016. Double *in situ* hybridization for microRNAs and mRNAs in brain tissues. *Frontiers in Molecular Neuroscience* **9**. DOI: 10.3389/fnmol.2016.00126.

Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, Nishida H, Yap CC, Suzuki M, Kawai J, Suzuki H, Carninci P, Hayashizaki Y, Wells C, Frith M, Ravasi T, Pang KC, Hallinan J, Mattick J, Hume DA, Lipovich L, Batalov S, Engström PG, Mizuno Y, Faghihi MA, Sandelin A, Chalk AM, Mottagui-Tabar S, Liang Z, Lenhard B, Wahlestedt C, RIKEN Genome Exploration Research Group, Genome Science Group (Genome Network Project Core Group), FANTOM Consortium. 2005. Antisense transcription in the mammalian transcriptome. *Science (New York, N.Y.)* **309**:1564–1566. DOI: 10.1126/science.1112009.

Keller W, Crouch R. 1972. Degradation of DNA RNA hybrids by ribonuclease H and DNA polymerases of cellular and viral origin. *Proceedings of the National Academy of Sciences of the United States of America* **69**:3360–3364.

Kempf VAJ, Trebesius K, Autenrieth IB. 2000. Fluorescent *in situ* hybridization allows rapid identification of microorganisms in blood cultures. *Journal of Clinical Microbiology* **38**:830–838.

Kernohan KD, Bérubé NG. 2014. Three dimensional dual labelled DNA fluorescent *in situ* hybridization analysis in fixed tissue sections. *MethodsX* **1**:30–35. DOI: 10.1016/j.mex.2014.04.001.

Kislauskis EH, Li Z, Singer RH, Taneja KL. 1993. Isoform-specific 3'-untranslated sequences sort alpha-cardiac and beta-cytoplasmic actin messenger RNAs to different cytoplasmic compartments. *The Journal of Cell Biology* **123**:165–172.

Lanfranco MF, Loane D, Mocchetti I, Burns M, Villapol S. 2017. Combination of fluorescent *in situ* hybridization (FISH) and immunofluorescence imaging for detection of cytokine expression in microglia/macrophage cells. *BIO-PROTOCOL* **7**. DOI: 10.21769/BioProtoc.2608.

Langenbacher AD, Rodriguez D, Di Maio A, De Tomaso AW. 2015. Whole-mount fluorescent *in situ* hybridization staining of the colonial tunicate *Botryllus schlosseri*. *Genesis (New York, N.Y.: 2000)* **53**:194–201. DOI: 10.1002/dvg.22820.

Lauter G, Söll I, Hauptmann G. 2011a. Multicolor fluorescent *in situ* hybridization to define abutting and overlapping gene expression in the embryonic zebrafish brain. *Neural Development* **6**:10. DOI: 10.1186/1749-8104-6-10.

Lauter G, Söll I, Hauptmann G. 2011b. Two-color fluorescent *in situ* hybridization in the embryonic zebrafish brain using differential detection systems. *BMC Developmental Biology* **11**:43. DOI: 10.1186/1471-213X-11-43.

Lederman L, Kawasaki ES, Szabo P. 1981. The rate of nucleic acid annealing to cytological preparations is increased in the presence of dextran sulfate. *Analytical Biochemistry* **117**:158–163. DOI: 10.1016/0003-2697(81)90705-3.

Legendre F, Cody N, Iampietro C, Bergalet J, Lefebvre FA, Moquin-Beaudry G, Zhang O, Wang X, Lécuyer E. 2013. Whole mount RNA fluorescent *in situ* hybridization of *Drosophila* embryos. *Journal of Visualized Experiments: JoVE*. DOI: 10.3791/50057.

Levsky JM, Shenoy SM, Pezo RC, Singer RH. 2002. Single-cell gene expression profiling. *Science* **297**:836–840.

Liu W, Zhang H, Xiang Y, Jia K, Luo M, Yi M. 2019. Molecular characterization of vasa homologue in marbled goby, *Oxyeleotris marmorata*: Transcription and localization analysis during gametogenesis and embryogenesis. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **229**:42–50. DOI: 10.1016/j.cbpb.2018.12.005.

Lyubimova A, Itzkovitz S, Junker JP, Fan ZP, Wu X, van Oudenaarden A. 2013. Single-molecule mRNA detection and counting in mammalian tissue. *Nature Protocols* **8**:1743–1758. DOI: 10.1038/nprot.2013.109.

Macnaughton SJ, O'Donnell AG, Embley TM. 1994. Permeabilization of mycolic-acid-containing actinomycetes for *in situ* hybridization with fluorescently labelled oligonucleotide probes. *Microbiology* **140**:2859–2865. DOI: 10.1099/00221287-140-10-2859.

Manz W, Wagner M, Amann R, Schleifer K-H. 1994. *In situ* characterization of the microbial consortia active in two wastewater treatment plants. *Water Research* 28:1715–1723.

Marra AN, Ulrich M, White A, Springer M, Wingert RA. 2017. Visualizing multiciliated cells in the zebrafish through a combined protocol of whole mount fluorescent in situ hybridization and immunofluorescence. *Journal of Visualized Experiments : JoVE*. DOI: 10.3791/56261.

Martindale MQ, Pang K, Finnerty JR. 2004. Investigating the origins of triploblasty: `mesodermal' gene expression in a diploblastic animal, the sea anemone

Nematostella vectensis (phylum, Cnidaria; class, Anthozoa). *Development* **131**:2463–2474. DOI: 10.1242/dev.01119.

McConaughy BL, Laird CD, McCarthy BJ. 1969. Nucleic acid reassociation in formamide. *Biochemistry* **8**:3289–3295. DOI: 10.1021/bi00836a024.

Meyer C, Garzia A, Tuschl T. 2017. Simultaneous detection of the subcellular localization of RNAs and proteins in cultured cells by combined multicolor RNA-FISH and IF. *Methods (San Diego, Calif.)* **118**–119:101–110. DOI: 10.1016/j.ymeth.2016.09.010.

Milman G, Langridge R, Chamberlin MJ. 1967. The structure of a DNA-RNA hybrid. *Proceedings of the National Academy of Sciences* **57**:1804–1810. DOI: 10.1073/pnas.57.6.1804.

Nakamura T, Nakamura T, Hamada H. 2013. Fluorescent 2 color whole mount *in situ* hybridization for a mouse embryo. *Protocol Exchange*. DOI: 10.1038/protex.2013.002.

Neufeld SJ, Zhou X, Vize PD, Cobb J. 2013. mRNA fluorescence *in situ* hybridization to determine overlapping gene expression in whole-mount mouse embryos. *Developmental Dynamics* **242**:1094–1100. DOI: 10.1002/dvdy.23993.

Oka Y, Sato TN. 2015. Whole-mount single molecule FISH method for zebrafish embryo. *Scientific Reports* **5**. DOI: 10.1038/srep08571.

Oliveira VC, Carrara RCV, Simoes DLC, Saggioro FP, Carlotti CG, Covas DT, Neder L. 2010. Sudan Black B treatment reduces autofluorescence and improves resolution of *in situ* hybridization specific fluorescent signals of brain sections. *Histology and Histopathology* **25**:1017–1024. DOI: 10.14670/HH-25.1017.

Oschwald R, Richter K, Grunz H. 1991. Localization of a nervous system-specific class II beta-tubulin gene in *Xenopus laevis* embryos by whole-mount *in situ* hybridization. *The International Journal of Developmental Biology* **35**:399–405.

Oxtoby E, Jowett T. 1993. Cloning of the zebrafish krox-20 gene (krx-20) and its expression during hindbrain development. *Nucleic Acids Research* **21**:1087–1095.

Pardue ML, Gall JG. 1969. Molecular hybridization of radioactive DNA to the DNA of cytological preparations. *Proceedings of the National Academy of Sciences of the United States of America* **64**:600–604.

Parker LM, Sayyadi N, Staikopoulos V, Shrestha A, Hutchinson MR, Packer NH. 2019. Visualizing neuroinflammation with fluorescence and luminescent lanthanide-based *in situ* hybridization. *Journal of Neuroinflammation* **16**:65. DOI: 10.1186/s12974-019-1451-2.

Pearson BJ, Eisenhoffer GT, Gurley KA, Rink JC, Miller DE, Sánchez Alvarado A. 2009. Formaldehyde-based whole-mount *in situ* hybridization method for planarians. *Developmental Dynamics* **238**:443–450. DOI: 10.1002/dvdy.21849.

Piette D, Hendrickx M, Willems E, Kemp CR, Leyns L. 2008. An optimized procedure for whole-mount *in situ* hybridization on mouse embryos and embryoid bodies. *Nature Protocols* **3**:1194–1201. DOI: 10.1038/nprot.2008.103.

Pinkel D, Landegent J, Collins C, Fuscoe J, Segraves R, Lucas J, Gray J. 1988. Fluorescence *in situ* hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. *Proceedings of the National Academy of Sciences* **85**:9138–9142.

Puskas RS, Manley NR, Wallace DM, Berger SL. 1982. Effect of ribonucleoside-vanadyl complexes on enzyme-catalyzed reactions central to recombinant DNA technology. *Biochemistry* **21**:4602–4608. DOI: 10.1021/bi00262a014.

Qi L, Knapton EK, Zhang X, Zhang T, Gu C, Zhao Y. 2017. Pre-culture sudan black B treatment suppresses autofluorescence signals emitted from polymer tissue scaffolds. *Scientific Reports* **7**:8361. DOI: 10.1038/s41598-017-08723-2.

Raj A, van den Bogaard P, Rifkin SA, van Oudenaarden A, Tyagi S. 2008. Imaging individual mRNA molecules using multiple singly labeled probes. *Nature methods* **5**:877–879. DOI: 10.1038/nmeth.1253.

Raj A, van Oudenaarden A. 2009. Single-molecule approaches to stochastic gene expression. *Annual Review of Biophysics* **38**:255–270. DOI: 10.1146/annurev.biophys.37.032807.125928.

Rich A. 1959. An analysis of the relation between DNA and RNA. *Annals of the New York Academy of Sciences* **81**:709–722. DOI: 10.1111/j.1749-6632.1959.tb49352.x.

Rich A. 1960. A hybrid helix containing both deoxyribose and ribose polynucleotides and its relation to the transfer of information between the nucleic acids. *Proceedings of the National Academy of Sciences of the United States of America* **46**:1044–1053.

Rink JC, Vu HT-K, Alvarado AS. 2011. The maintenance and regeneration of the planarian excretory system are regulated by EGFR signaling. *Development* **138**:3769–3780. DOI: 10.1242/dev.066852.

Rocha R, Almeida C, Azevedo NF. 2018. Influence of the fixation/permeabilization step on peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) for the detection of bacteria. *PLOS ONE* **13**:e0196522. DOI: 10.1371/journal.pone.0196522.

719 **Rudkin GT, Stollar BD.** 1977. High resolution detection of DNA–RNA hybrids in situ by
720 indirect immunofluorescence. *Nature* **265**:472–473. DOI: 10.1038/265472a0.
721 **Sarkar S, Hopper AK.** 1998. tRNA nuclear export in *Saccharomyces cerevisiae*: In situ
722 hybridization analysis. *Molecular Biology of the Cell* **9**:3041–3055. DOI:
723 10.1091/mbc.9.11.3041.
724 **Schiemann SM, Martín-Durán JM, Børve A, Vellutini BC, Passamaneck YJ, Hejnol A.**
725 2017. Clustered brachiopod Hox genes are not expressed collinearly and are
726 associated with lophotrochozoan novelties. *Proceedings of the National Academy of*
727 *Sciences of the United States of America* **114**:E1913–E1922. DOI:
728 10.1073/pnas.1614501114.
729 **Schurter MJ, LeBrun DP, Harrison KJ.** 2002. Improved technique for fluorescence *in situ*
730 hybridisation analysis of isolated nuclei from archival, B5 or formalin fixed, paraffin
731 wax embedded tissue. *Molecular Pathology* **55**:121–124.
732 **Sepsi A, Fábián A, Jäger K, Heslop-Harrison JS, Schwarzacher T.** 2018. ImmunoFISH:
733 Simultaneous visualisation of proteins and DNA sequences gives insight into meiotic
734 processes in nuclei of grasses. *Frontiers in Plant Science* 9. DOI:
735 10.3389/fpls.2018.01193.
736 **Shaffer SM, Wu M-T, Levesque MJ, Raj A.** 2013. Turbo FISH: A method for rapid single
737 molecule RNA FISH. *PLoS ONE* 8. DOI: 10.1371/journal.pone.0075120.
738 **Shalek AK, Satija R, Shuga J, Trombetta JJ, Gennert D, Lu D, Chen P, Gertner RS,**
739 **Gaublomme JT, Yosef N, Schwartz S, Fowler B, Weaver S, Wang J, Wang X, Ding**
740 **R, Raychowdhury R, Friedman N, Hacohen N, Park H, May AP, Regev A.** 2014.

Single-cell RNA-seq reveals dynamic paracrine control of cellular variation. *Nature* **510**:363–369. DOI: 10.1038/nature13437.

Shiura H, Okamoto A, Sasaki H, Abe K. 2014. Whole-mount MeFISH: A novel technique for simultaneous visualization of specific DNA methylation and protein/RNA expression. *PLOS ONE* 9:e95750. DOI: 10.1371/journal.pone.0095750.

Singer RH, Ward DC. 1982. Actin gene expression visualized in chicken muscle tissue culture by using *in situ* hybridization with a biotinated nucleotide analog. *Proceedings of the National Academy of Sciences of the United States of America* **79**:7331–7335.

Singh L, Jones KW. 1984. The use of heparin as a simple cost-effective means of controlling background in nucleic acid hybridization procedures. *Nucleic Acids Research* **12**:5627–5638.

Sinigaglia C, Thiel D, Hejnol A, Houliston E, Leclère L. 2018. A safer, urea-based *in situ* hybridization method improves detection of gene expression in diverse animal species. *Developmental Biology* **434**:15–23. DOI: 10.1016/j.ydbio.2017.11.015.

Steinmetz PRH, Kostyuchenko RP, Fischer A, Arendt D. 2011. The segmental pattern of otx, gbx, and Hox genes in the annelid *Platynereis dumerilii*. *Evolution & Development* **13**:72–79. DOI: 10.1111/j.1525-142X.2010.00457.x.

S**zabo Q, Jost D, Chang J-M, Cattoni DI, Papadopoulos GL, Bonev B, Sexton T, Gurgo J, Jacquier C, Nollmann M, Bantignies F, Cavalli G.** 2018. TADs are 3D structural units of higher-order chromosome organization in *Drosophila*. *Science Advances* **4**:eaar8082. DOI: 10.1126/sciadv.aar8082.

763 **Taniguchi Y, Choi PJ, Li G-W, Chen H, Babu M, Hearn J, Emili A, Xie XS.** 2010.
764 Quantifying *E. coli* proteome and transcriptome with single-molecule sensitivity in
765 single cells. *Science (New York, N.Y.)* **329**:533–538. DOI: 10.1126/science.1188308.
766 **Tanner M, Gancberg D, Di Leo A, Larsimont D, Rouas G, Piccart MJ, Isola J.** 2000.
767 Chromogenic *in situ* hybridization. *The American Journal of Pathology* **157**:1467–
768 1472.
769 **Tessmar-Raible K, Steinmetz PRH, Snyman H, Hassel M, Arendt D.** 2005. Fluorescent
770 two-color whole mount *in situ* hybridization in *Platynereis dumerilii* (Polychaeta,
771 Annelida), an emerging marine molecular model for evolution and development.
772 *BioTechniques* **39**:460–464. DOI: 10.2144/000112023.
773 **Thavarajah R, Mudimbaimannar VK, Elizabeth J, Rao UK, Ranganathan K.** 2012.
774 Chemical and physical basics of routine formaldehyde fixation. *Journal of Oral and*
775 *Maxillofacial Pathology: JOMFP* **16**:400–405. DOI: 10.4103/0973-029X.102496.
776 **Thiruketheeswaran P, Kiehl E, D'Haese J.** 2016. Soluble calcium-binding proteins
777 (SCBPs) of the earthworm *Lumbricus terrestris*: molecular characterization and
778 localization by FISH in muscle and neuronal tissue. *Histochemistry and Cell Biology*
779 **146**:635–644. DOI: 10.1007/s00418-016-1463-2.
780 **Titford M.** 2001. Safety considerations in the use of fixatives. *Journal of Histotechnology*
781 **24**:165–171. DOI: 10.1179/his.2001.24.3.165.
782 **Volpi EmanuelaV.** 2017. Formamide-free fluorescence *in situ* hybridization (FISH). In:
783 Liehr T ed. *Fluorescence in situ hybridization (FISH)*. Springer Protocols Handbooks.
784 Springer Berlin Heidelberg, 135–139. DOI: 10.1007/978-3-662-52959-1_12.

Volpi EV, Bridger JM. 2008. FISH glossary: an overview of the fluorescence *in situ* hybridization technique. *BioTechniques* **45**:385–386, 388, 390 passim. DOI: 10.2144/000112811.

Wang W, Tang Y, Li J, Jiang L, Jiang Y, Su X. 2015. Detection of ALK rearrangements in malignant pleural effusion cell blocks from patients with advanced non-small cell lung cancer: A comparison of Ventana immunohistochemistry and fluorescence *in situ* hybridization. *Cancer Cytopathology* **123**:117–122. DOI: 10.1002/cncy.21510.

Warheit DB, Kinney LA, Carakostas MC, Ross PE. 1989. Inhalation toxicity study of formamide in rats. *Toxicological Sciences* **13**:702–713. DOI: 10.1093/toxsci/13.4.702.

Wiegant J, Bezrookove V, Rosenberg C, Tanke HJ, Raap AK, Zhang H, Bittner M, Trent JM, Meltzer P. 2000. Differentially painting human chromosome arms with combined binary ratio-labeling fluorescence *in situ* hybridization. *Genome Research* 10:861–865.

Wyeth RC, Croll RP. 2011. Peripheral sensory cells in the cephalic sensory organs of *Lymnaea stagnalis*. *The Journal of Comparative Neurology* **519**:1894–1913. DOI: 10.1002/cne.22607.

Yoon DS, Pendergrass DL, Lee M-H. 2016. A simple and rapid method for combining fluorescent *in situ* RNA hybridization (FISH) and immunofluorescence in the *C. elegans* germline. *MethodsX* **3**:378–385. DOI: 10.1016/j.mex.2016.05.001.

Zenklusen D, Singer RH. 2010. Analyzing mRNA Expression using single mRNA resolution fluorescent *in situ* hybridization. *Methods in enzymology* **470**:641–659. DOI: 10.1016/S0076-6879(10)70026-4.

808 **Zhang Y, Liu XS, Liu Q-R, Wei L.** 2006. Genome-wide *in silico* identification and analysis of
809 cis natural antisense transcripts (cis-NATs) in ten species. *Nucleic Acids Research*
810 **34**:3465–3475. DOI: 10.1093/nar/gkl473.
811

Figure 1

Schematic representation of the technological development of fluorescent *in situ* hybridization.

[1]Pardue & Gall, 1969; [2]Gall & Pardue, 1969; [3]Rudkin & Stollar, 1977; [4]Tanner et al., 2000 ;

[5]Singer & Ward, 1982; [6]Femino et al., 1998; [7]Raj et al., 2008.

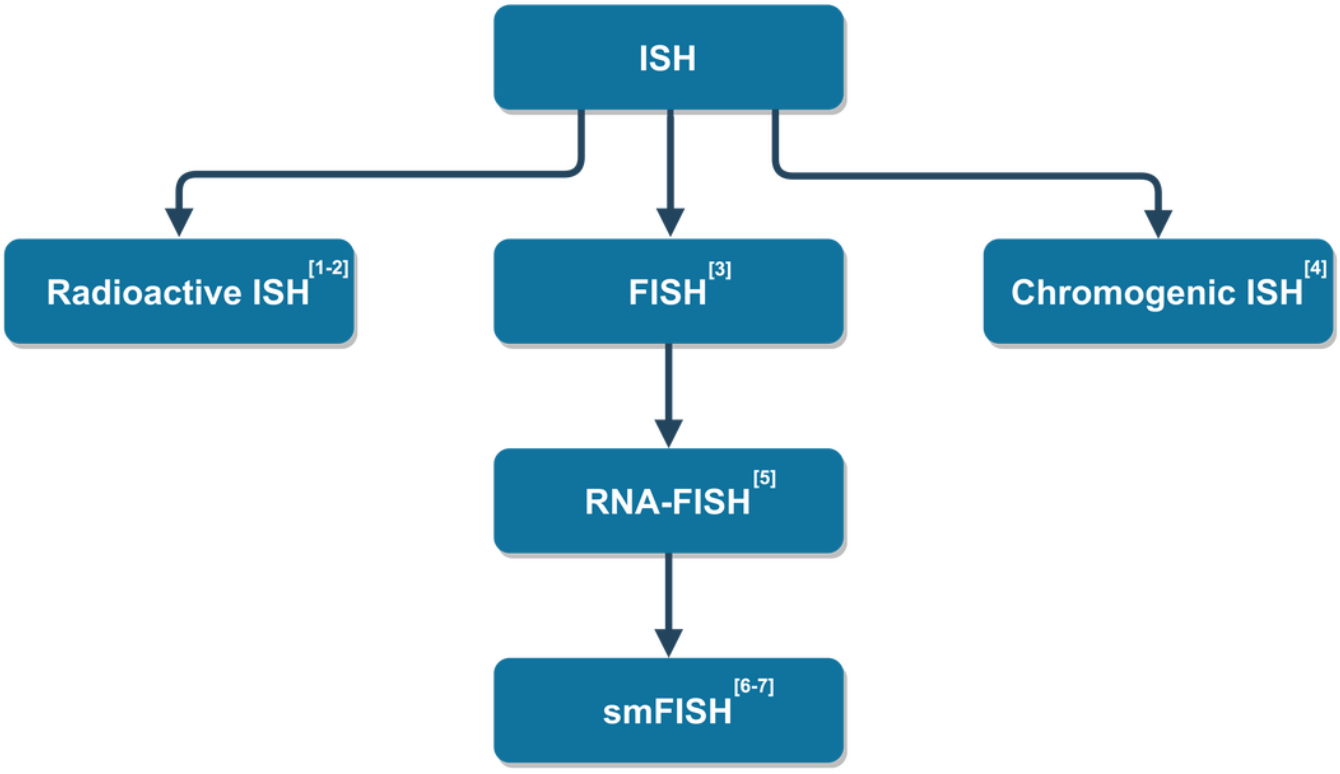

Figure 2

Schematic representation of the riboprobe and oligonucleotide *in situ* hybridization probe types.

A) Hapten-labeled RNA probes must be bound by an antibody labeled with a fluorophore to allow for visualization. B) DNA oligomers directly labeled with a fluorophore can be directly visualized.

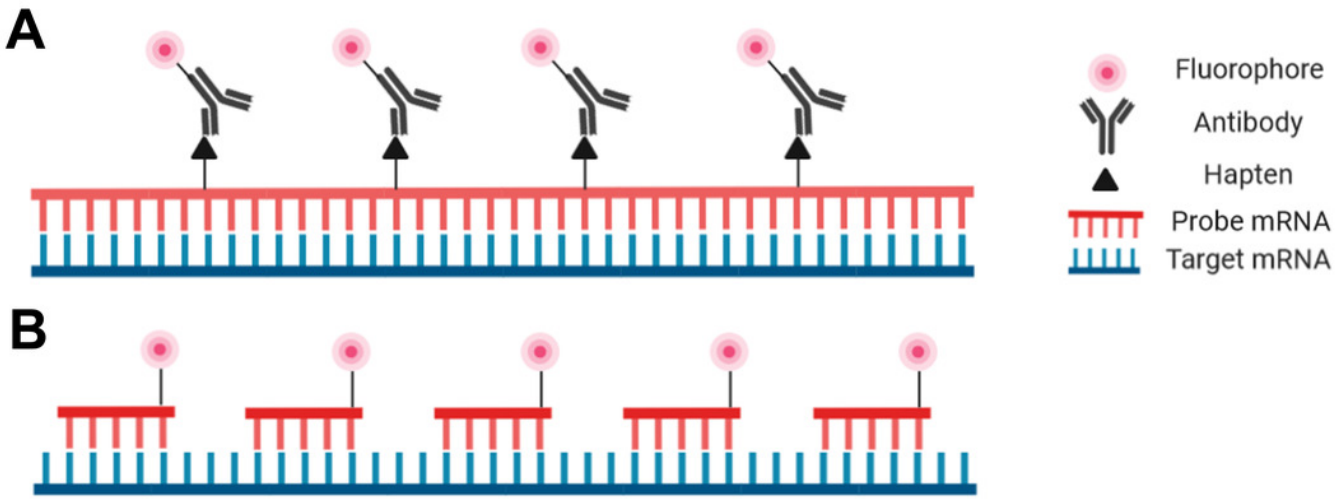

Table 1(on next page)

Representative panel of fluorescence *in situ* hybridization (FISH) tissue preparation protocols on a diverse group of tissues types.

Special attention is paid to the fixation, permeabilization, acetylation, and post-fixation strategies.

Table 1. Representative panel of fluorescence *in situ* hybridization (FISH) tissue preparation protocols on a diverse group of tissues types. Special attention is paid to the fixation, permeabilization, acetylation, and post-fixation strategies.

Author(s)	**Singer and Ward 1982**	**Oka and Sato 2015**	**Langenbacher et al. 2015**	**Martín-Durán et al. 2016**	**Thiruketheeswaran et al. 2016**	**Parker et al. 2019**	**Liu et al. 2019**
Sample	Chicken muscle	Zebrafish embryo	Tunicate	Brachiopod embryos	Earthworm muscle and neuronal tissue	Microglia and macrophages	Marbled goby fish gametes
Preparation	Cultured cells	Whole mount	Whole mount	Whole mount	Tissue sections	Cultured cells	Tissue sections
Fixation	PFA (2%), 1 min	PFA (4%), overnight	FA (4%), 3 h	FA (3.7%), 4 h at 4˚C	FA (3.7%), 10 min	FA (4%), 10 min	PFA (4%), overnight
Permeabilization	HCl (0.1 M), 10 min			Methanol (100%), for storage		Ethanol (70%), 30 min	
Proteinase	Proteinase K (50 µg/mL), 10 min		Proteinase K (10 µg/mL), 30 min	Proteinase K (10 µg/mL), 3 min			Proteinase K (10 µg/mL), 5 min
Acetylation	Acetic acid (0.25%)			Acetic anhydride (0.3%)			
Post-fixation	PFA (4%), 10 min		FA (4%), 20 min	FA (3.7%), 1 h			PFA (4%), 30 min

Table 2(on next page)

Representative panel of fluorescence *in situ* hybridization (FISH) hybridization protocols on a diverse group of tissues types.

Special attention is paid to the type of probe used, as well as the ingredients of the hybridization buffer.

Table 2. Representative panel of fluorescence *in situ* hybridization (FISH) hybridization protocols on a diverse group of tissues types.

Special attention is paid to the type of probe used, as well as the ingredients of the hybridization buffer.

Author(s)	**Singer and Ward 1982**	**Oka and Sato 2015**	**Langenbacher et al. 2015**	**Martín-Durán et al. 2016**	**Thiruketheeswaran et al. 2016**	**Parker et al. 2019**	**Liu et al. 2019**
Sample	Chicken muscle	Zebrafish embryo	Tunicate	Brachiopod embryos	Earthworm muscle and neuronal tissue	Microglia and macrophages	Marbled goby fish gametes
Preparation	Cultured cells	Whole mount	Whole mount	Whole mount	Tissue sections	Cultured cells	Tissue sections
Probe type	300 nt DNA	20 nt oligonucleotides	1000 nt RNA	1000 nt RNA	20 nt oligonucleotides	600 nt RNA	1000 nt RNA
Ingredients	Probe	Probe	Probe	Probe	Probe	Probe	Probe
	Formamide (50%)	Formamide (10%)	Formamide (65%)	Formamide (50%)	Formamide (10%)	Formamide (50%)	Formamide (50%)
	Herring sperm DNA (0.5 µg/µL)			Salmon sperm DNA (0.1 µg/µL)		Herring sperm DNA (250 µg/mL)	
	tRNA (2 µg/µL)		Torula yeast RNA (5 mg/mL)	tRNA (0.1 µg/µL)			tRNA (500 µg/mL)
	SSC	SSC	SSC	SSC	SSC	SSC	SSC

	BSA (10 mg/mL)	BSA (2 mg/mL)			BSA (1 mg/mL)		
	Vanadyl sulfate (10 mM)	Vanadyl ribonucleoside complex (2 mM)					
	Dextran sulfate (100 mg/mL)	Dextran sulfate (100 mg/mL)	Heparin (50 µg/mL)	Heparin (50 µg/mL)	Dextran sulfate (100 mg/mL)	Dextran sulfate (50 mg/mL)	Heparin (50 µg/mL)
		Triton X-100 (0.1%)	Tween-20 (0.1%)	Tween-20 (0.1%) and SDS (1%)		Tween-20 (0.1%)	Tween-20 (0.1%)
			Denhardt's solution			Denhardt's solution	
Temp.	34ºC	30ºC	58-65ºC	65ºC	30ºC	37ºC	60 ºC
Time	48-72 h	Overnight	Overnight	24-60 h	Overnight	16 h	16 h

3

4

Table 3(on next page)

Representative panel of fluorescence *in situ* hybridization (FISH) post-hybridization protocols on a diverse group of tissues types.

As post-hybridization washes are generally numerous and become incrementally more stringent with each wash, not every wash step is documented here. However, the composition of the initial washes and the final washes are highlighted.

Table 3. Table 2. Representative panel of fluorescence *in situ* hybridization (FISH) post-hybridization protocols on a diverse group of tissues types. As post-hybridization washes are generally numerous and become incrementally more stringent with each wash, not every wash step is documented here. However, the composition of the initial washes and the final washes are highlighted.

Author(s)	**Singer and Ward 1982**	**Oka and Sato 2015**	**Langenbacher et al. 2015**	**Martín-Durán et al. 2016**	**Thiruketheeswaran et al. 2016**	**Parker et al. 2019**	**Liu et al. 2019**
Sample	Chicken muscle	Zebrafish embryo	Tunicate	Brachiopod embryos	Earthworm muscle and neuronal tissue	Microglia and macrophages	Marbled goby fish gametes
Preparation	Cultured cells	Whole mount	Whole mount	Whole mount	Tissue sections	Cultured cells	Tissue sections
Initial washes:	Formamide (50%) and SSC	Formamide (10%), SSC and Triton X-100 (0.1%)	Unreported	Hybridization buffer and SSC	Formamide (10%) and SSC, 30 min	SSC buffer	SSC buffer and Tween-20 (0.1%)
Final washes:	PBS, 1h	SSC		SSC and PBS with Tween-20		PBS with Tween-20	Maleic acid buffer and Tween-20 (0.1%)